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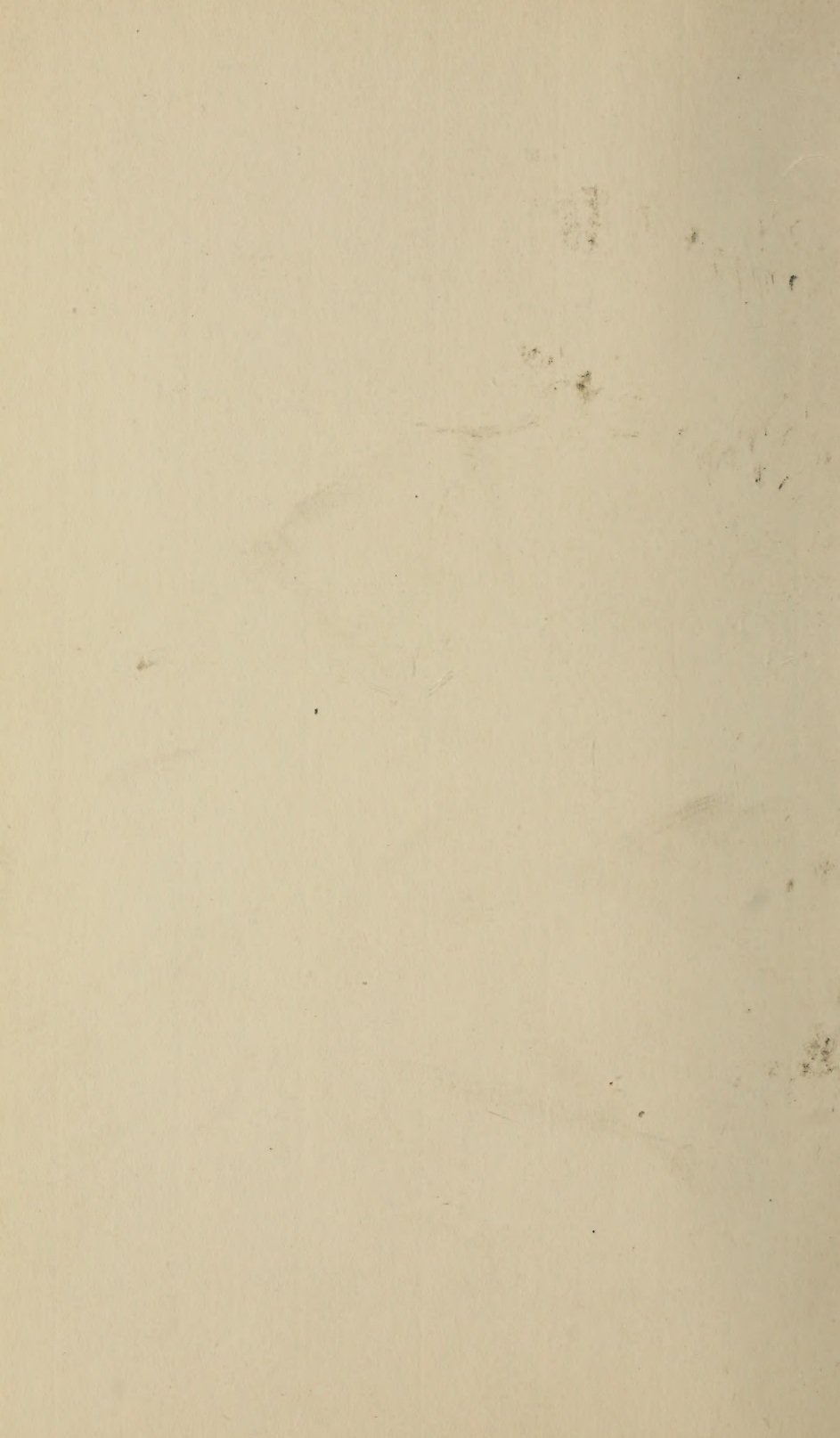
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A STUDY OF EXPERIMENTAL DIABETES IN THE CANINE AND ITS RELATION TO HUMAN DIABETES.*

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INTRODUCTION:

The importance of the study of experimentally-produced diseases in animals; Diabetes a failure to utilize dextrose; The elusiveness of its etiology and pathology; Possible relationship of diabetes to disease of the pancreas; The finer anatomy of the pancreas; Degenerate processes already observed in the diabetic pancreas, notably in the islands of Langerhans; Review of previous experimental observations, attributing to the islands an internal secretion vital to the utilization of dextrose.

THE WRITER'S OBSERVATIONS UPON EXPERIMENTAL DIABETES:

Relating to functional activity and degeneration in the islands of Langerhans; Terminal "hydropic" degeneration in the islands; Earlier changes suggestive of functional activity, followed by exhaustion of the island cells; Changes suggestive of island regeneration.

Relating to the physical effect upon the islands of Langerhans of the ingestion of carbohydrate food; The conversion of mild glycosuria into fatal diabetes; Progressive anatomical changes in the islands, accompanying this process.

Relating to ligation of the pancreatic ducts and the dietetic administration of fresh pancreas; Duct ligation may bring about a cessation of glycosuria in experimental diabetes; The administration of fresh pancreas with the food causes a return of glycosuria; Degeneration of the islands of Langerhans under these conditions.

Relating to the effect of certain nervous stimuli upon glycosuria, and upon the appearance of the islands; Is experimental diabetes primarily a failure of glycogenesis or glycolysis? Release of dextrose from the liver

* Received for publication May 10, 1915.

by stimulation of the splanchnic nerves; Rough tests of the waste of dextrose (failure of glycolysis) under these conditions, when (1) the pancreas is intact, (2) reduced one-half, or (3) reduced four-fifths; A considerable reduction of the pancreas may cause splanchnic stimulation to be more promptly effective, but does not appear to cause a failure of glycolysis; Absence of physical changes in the islands in the experiments.

DISCUSSION OF EXPERIMENTAL DIABETES:

The physiological significance of the progressive alterations in the islands in experimental diabetes; The relation of these changes to the pathology of diabetes in man; The relation of the clinical course of experimental diabetes to that of the disease in man. The function of the island secretion probably glycogenic; Recapitulation of the pathologic, etiologic, and clinical relations of human and experimental diabetes.

APPENDIX:

Protocols of experimental observations; histological technic.

INTRODUCTION. — It is becoming increasingly apparent that the etiology of a multitude of unconquered diseases is not to be found in post-mortem pathology, and as the realization of the deceptiveness of terminal alterations has crystallized, pathologists, physiologists, and clinicians alike have turned for light to the study of abnormal physiology in living beings. This study may be and has been carried far in man, but it is rather in the experimentally-produced and readily-controlled diseases of animals that we are now seeking for the answer to the riddles of human pathology. When once by the older methods we can discover in any one organ or set of organs structural changes clearly responsible for familiar symptoms, we are then able to make further progress by the more modern route, but when, on the other hand, we are unable to connect the outward manifestation of disease with any known anatomical structures, the experimental method gropes and fails. Such for many years has been the case with diabetes.

It is fair to say that the only definitely-settled feature of this disease is the inability of the body to utilize dextrose, the essential carbohydrate food of the tissues. The disease is, then, a functional one, but characterized by definite symptoms and a progressive course. The body cannot store carbohydrate for its own use. The tissues cannot burn

it in the form in which it is present in the body fluids. It is excreted with diuretic effect through the kidneys, while the body wastes by consuming its own vital materials. Obviously, then, such a disease must be associated with abnormal physiological processes on the part of many organs. The liver is the chief storage depot for glycogen, yet Claude Bernard's hypothesis, that the liver is the organ primarily responsible for diabetes, can easily be disproved. The presence in the circulation of an excess of the secretion of the adrenal medulla causes hyperglycemia and glycosuria by glycogenolytic action upon the liver, yet the action of adrenalin has been shown to depend upon the integrity of the sympathetic nerve-supply to this organ, and it is strongly suggested that the adrenal is merely a sort of activator for the dextrose-releasing mechanism which resides in the sympathetic system. Nor are the adrenals the seat of any recognized terminal pathological alterations in diabetes.

Many drugs, sensory stimuli, and what in general may be called nervous influences, may cause glycosuria, yet these abnormal factors do not prevent the body from utilizing dextrose, and the conditions to which they give rise are neither progressive nor permanent. Claude Bernard's *piqûre* brings about an intense glycosuria, but though the wound in the floor of the fourth ventricle never heals, the glycosuria, as in other perhaps similar traumatic conditions, is transient. Finally, disorders of certain ductless glands may be accompanied by a discharge of dextrose in the urine, yet the glycosuria of pituitary disease does not run the course of diabetes, and the low carbohydrate tolerance of exophthalmic goitre seems merely a by-effect of the nervous phenomena of hyperthyroidism.

The pancreas and diabetes. — During the nineteenth century the attention of pathologists was attracted to the pancreas by reason of the occasional association of diabetes with gross alterations, amounting in some instances to destruction, of this organ. An Englishman, Cowley,¹ reported an instance of this association in 1788, and as early as 1845, Bouchardat,²

in seeking to demonstrate the significance of pancreatic changes in diabetes, attempted to excise the gland in living dogs. Careful studies of gross pathological material, such as were reported by Lancereaux³ in 1877, gave rise to the hope that in derangements of the pancreas the cause of diabetes had indeed been found. Subsequent microscopical studies now strengthened, now seemed to discredit this probable relationship, and only recently has the discovery of minute degenerative changes in the pancreas thrown the weight of evidence in its favor.

Anatomy. — In order to make intelligible a discussion of these finer alterations in the pancreas of diabetics, a brief sketch of its microscopic anatomy is appended.

For a minute description of the pancreas, more especially as regards the secretory cells and their distribution, the reader is referred to Bensley's illuminating work⁴ upon the pancreas of the guinea-pig. The pancreas contains two entirely distinct varieties of secreting cells: the acinous cells which form zymogen, the precursor of the digestive secretion poured into the intestine; and the cells of the islands of Langerhans, which secrete a substance presumably taken into the portal circulation. Both tissues are shown in the accompanying illustrations, Figures 1 and 2. A system of ducts, of which the fine terminal ramifications are called centro-acinar cells, drains the acini. The islands as well are associated with ducts of a different sort — primitive structures from which the islands bud in fetal life, and which carry no known secretion. Many of the cells of both varieties of ducts resemble the cells of the islands. They contain similar granules and possibly constitute potential island cells.

The islands themselves contain cells of two sorts — the "A" and "B" cells of Lane⁵ and Bensley,⁴ characterized by definite reactions to fixatives and stains. In the preparation from which Figures 1 and 2 are drawn, the granules of the "A" cells take a bright red stain, while the "B" cells stain a slate blue. The "B" cells far outnumber the "A," and in the resting island both are crowded together in such

a way that their outlines are indistinct. Finally, like all other secreting cells, those of the pancreas contain mitochondria, rod-shaped structures having a strong affinity for fuchsin, and not to be confused with secreting granules. Inasmuch as they become more noticeable as cell-activity increases, considerable importance is attached to their appearance.

The pathological alterations to which reference has already been made concern principally the islands of Langerhans. They may be said to be of three kinds: *acute degeneration*, *chronic degeneration*, and *regeneration*. The first, or acute form, was first described as "hydropic" degeneration by Weichselbaum,^{6,7} who finds it in fifty per cent of several hundred autopsies upon diabetics of all ages. In those individuals dying before the age of forty, nearly eighty per cent exhibit this peculiar degeneration. The change occurs entirely within the islands, and appears to be due to the absorption of fluid by the island cells, which swell, lose their characteristic staining reaction, and ultimately collapse and disappear. Weichselbaum accounts in this way for the extraordinary diminution in the number of islands so often noted in diabetes. Although hydropic degeneration of the islands is a recent discovery, it has often been shown by him to be associated with more chronic degenerative changes described by other investigators.

These chronic forms of degeneration have been most minutely investigated by Opie,^{8,9} who has done more than any one else to connect with diabetes, hyaline and sclerotic degeneration in the islands of Langerhans, and in the pancreas in general. He has described extensive sclerosis of the pancreas, involving alike acini and islands, and not uncommonly hyaline and other forms of degeneration in the islands alone. It remains true, however, that extensive destruction of both islands and acini has often been found in individuals altogether free from diabetes, and it cannot be asserted upon the evidence of post-mortem pathology that chronic forms of pancreatic degeneration are pathognomonic of the disease.

Finally, regenerative changes in the islands have been

noted by Weichselbaum,⁷ Cecil,¹⁰ MacCallum,¹¹ and others, and this even as an accompaniment of degeneration. The phenomenon is quite creditable in view of Bensley's demonstration of large numbers of potential island cells in ducts, and in view of the embryological origin of the islands from these ducts; but inasmuch as the evidence of regeneration is based upon the appearance of cells exposed to post-mortem alteration, too much reliance must not be placed upon it.

This brief summary presents, then, the case for those contending that diabetes is at least associated with pathological changes in the pancreas. That investigators have generally accepted this view is shown by the trend of experimental research, and it has been demonstrated that of all organs the pancreas has the most vital influence upon the complicated mechanism of carbohydrate metabolism.

The results of experimental and clinical studies may be most conveniently indicated under the following headings:

- A. Evidence that some portion of the pancreas is necessary to life and to the utilization of dextrose.
- B. Evidence that the pancreas contributes an internal secretion necessary to the utilization of dextrose.
- C. Evidence that the islands of Langerhans supply the internal secretion of the pancreas.

A. That some portion of the pancreas is necessary to life and the utilization of dextrose has been conclusively demonstrated. Minkowski's¹² classical experiments proved that the removal of the entire pancreas invariably subjects an animal to a high degree of glycosuria, rapid wasting, and death. It has since been shown by Sandmeyer¹³ and others that the removal of all but a small portion of the pancreas is compatible with health, but that if this portion is subjected to a gradual sclerosis, as by a loss of its blood-supply and the damming back of its external secretion, a condition similar to that brought about by total pancreatectomy will gradually ensue. The depancreatized animal, during the

remainder of its life, may be said to be in a condition similar to that occasionally seen in man, when diabetes follows acute pancreatitis in such a way as to suggest a gradual destruction of the organ by the invasion of scar tissue.

B. That the pancreas contributes an internal secretion necessary to the utilization of dextrose is almost equally certain, and the most perfect experimental demonstration of this function is obtained by the graft experiment employed by Minkowski,¹⁴ Hédon,¹⁵ Lombroso,¹⁶ and Thiroloix.¹⁷ In this experiment all of the pancreas, except a small portion of the duodenal end, is excised. This portion, the uncinat process, possesses a blood-supply altogether independent of the vessels passing to the intestine. It is separated from the intestine and transplanted, with its own vessels intact, into the abdominal wall. From this situation the external secretion (pancreatic juice) escapes to the surface of the body, and the animal remains in good health. After a few weeks the blood (and nerve) supply to the transplant is divided, but owing to the establishment of a capillary circulation, the transplant lives, and again the animal remains in good health. Finally, the graft is excised and experimental diabetes at once sets in. This observation disposes of Pflüger's contention that the disturbance of the nerve-supply to the duodenum is the cause of diabetes following pancreatectomy, and furnishes evidence that the external secretion of the pancreas is not essential to life. It does not indicate whether the internal secretion is supplied by the acinous or island cells, but brings positive evidence that the pancreas contributes to the circulation a substance necessary to the utilization of dextrose.

C. The evidence that the islands of Langerhans supply this internal secretion is strong and has been generally accepted. It has been demonstrated, accidentally in man and purposefully in animals, that complete destruction of the acinous tissue alone is not followed by diabetes. More than this, it appears from experiments of Laguesse,¹⁸ Kirkbride,¹⁹ MacCallum,²⁰ and others, that in those instances in which ligation of the pancreatic duct is followed by a high degree of

pancreatic atrophy, the islands remain well-preserved while the acini are totally destroyed. In the only recorded instance in which the remaining island tissue has subsequently been removed, diabetes has followed (MacCallum). It has been established, moreover, that the cells of the islands contain abundant granules of a secretion which has no known external outlet, and that the islands from an embryological standpoint are independent structures. There is no indication, on the other hand, that the acinous cells have any but an external secretion.

It may, therefore, be assumed to be true that the islands of Langerhans secrete a substance, without which the individual becomes diabetic in the sense that it is subject to gradual wasting, together with glycosuria and polyuria, and that eventually it dies. In both experimental and human diabetes the body is unable to store or fix glycogen from dextrose or to consume dextrose, and in both there is hyperglycemia. In the acute experimental disease, however, acetone and diacetic acid are rarely seen in the urine, and the animal is not subject to diabetic coma.

If, then, lack of the internal secretion of the pancreas gives rise to a condition so closely simulating diabetes, may we not learn more of this secretion by studying the physical changes in this organ in the presence of the experimental disease? And if the conditions underlying the production of the secretion are understood, is it not possible so to modify them as to obtain more knowledge of the factors concerned with human diabetes? In answer to these questions, the artificial disease can be induced without entirely removing the pancreas, leaving for minute study the tissues which manufacture the secretion. It is possible to trace in these tissues changes corresponding to the activity or loss of the secretion, and to induce in response to a number of external stimuli such as may well be present in human diabetes, responsive changes in the islands of Langerhans.

EXPERIMENTAL OBSERVATIONS. — The ground covered by this investigation includes, first, the observation of physical

changes in the islands of Langerhans corresponding to secretory activity and degeneration, and second, a study of the conditions under which activity or degeneration are called forth. The experiments are grouped under the following four heads:

I. Those demonstrating various stages of functional activity and degeneration in the islands of Langerhans.

II. Those demonstrating the physical effect upon the islands of Langerhans of administering carbohydrate food to an animal in whom the greater part of the pancreas has been removed, with the conversion of a mild glycosuria into permanent experimental diabetes.

III. Those demonstrating the physical effect upon the islands of Langerhans of duct ligation and the dietetic administration of fresh pancreas to an animal with a much reduced amount of pancreatic tissue.

IV. The effect of certain nervous stimuli upon glycosuria and upon the physical character of the islands.

I. Functional activity and degeneration in the islands. — (Experiments³² reported elsewhere demonstrating various stages of functional activity were originally performed by the writer upon the cat, with results similar to those obtained independently by Allen.²¹ The accuracy of these earlier observations has been confirmed by the results of recent experiments, which are reported here to introduce more complete microscopic studies.) Dogs previously free from glycosuria are subjected to the removal, as closely as can be estimated, of nine-tenths of the pancreas. (This proportion has been determined empirically for dogs by both Allen and myself. In cats the proportion which must be removed is nearer five-sixths. In either animal it is subject to considerable variation.) The operation leaves only that portion of the gland immediately adjacent to the principal secretory duct, and interferes in no way with the blood-supply of the remaining fragment, or of the duodenum. The external secretion, though considerably diminished in amount, has free access to the intestine, and, indeed, the relation of this bit of pancreas to both the bowel and to the mesentery is practically undisturbed. Animals recover rapidly from the operation, which is accompanied by neither loss of blood nor

shock. From the day of operation the condition of the animal depends upon whether sufficient pancreas has been removed to bring on glycosuria. There is often some sugar in the urine during the twelve hours following the operation, but if so little pancreas has been removed that glycosuria soon ceases upon a meat diet, more of the gland is excised at a second operation.

Under the proper conditions of the experiment the animal is now rendered glycosuric upon a meat diet, and is said to suffer, according to the nomenclature of Allen,²¹ from "diabetes gravis." He appears well, is lively and active, and his appetite is voracious, but he soon begins to lose weight and continues gradually to deteriorate until his death. Glycosuria is never absent. It begins mildly, increases in severity, and only diminishes slightly in the last days of life. Diabetic coma, as we see it in humans, does not occur, and acetone and diacetic acid rarely appear in the urine. Most experiments of this series have been cut short, even while the animal was still active though emaciated, but it is probable that none of the dogs could have lived over two months after the establishment of the disease.

It is not to be supposed, however, that experimental diabetes is not subject to considerable variation. An animal will sometimes remain for some days or weeks on the edge, as it were, of a dangerous breakdown. In such a case polyuria is not a prominent symptom, and one can foresee the end when the output of urine is rapidly increased. On the other hand, sugar may disappear from the urine after a mild form of the disease has existed for some time. Several animals in the series have recovered spontaneously and have been used for further observations. In one instance a fairly severe diabetes continued for some weeks upon a meat diet, and in spite of an intercurrent distemper seemed finally to be diminishing in severity, as was shown by the diminution of polyuria and glycosuria, but the eventual loss of weight and strength seemed to necessitate the sacrifice of the animal.

Terminal changes in experimental diabetes. — The post-mortem examination of diabetic animals discloses consistent pathological changes only in the remaining fragment of pancreas. The gross appearance of the gland is not remarkable, though hypertrophy in young animals is frequently observed, and on microscopic examination the acinous tissue is normal and presents its usual content of zymogen. Remarkable alterations, however, are noticeable in the islands of Langerhans (Figures 5, 6, and 7). For the most part these structures, usually dense masses of closely-packed cells, have the appearance of irregular, coarse-meshed sieves. The cell outlines, the shrunken nucleus, a few mitochondrial filaments attached to fine shreds of protoplasm alone are left. The secretory granules have disappeared. These changes take place only in the "B" cells of Lane and Bensley, for in a careful microscopic study of many hundreds of sections no alterations in the "A" cells have ever been noted. Inasmuch, however, as the "B" cells far outnumber the "A," the preservation of the latter does not materially affect the stricken appearance of the islands. This alteration is most easily distinguished in specimens in which secretory granules are preserved and stained, but it may readily be seen in preparations fixed in Zenker's fluid and stained by routine methods. These appearances are obviously the counterpart of Weichselbaum's "hydropic" degeneration observed in human diabetes.

Such changes are universally apparent in the pancreas of animals subjected to a rapidly-fatal experimental diabetes. In instances of a less rapid manifestation of the disease, it is easy to see how islands may disappear or become altogether unrecognizable. A mass of degenerate empty cells collapses, probably by the absorption of the fluid which the swollen cells contain, and only the scattered, well-preserved "A" cells remain to identify the island (Figure 7). In some instances scar tissue seems to be invading these degenerate structures.

Functional and early degenerative changes in experimental diabetes. — The terminal lesions already described

are sufficiently remarkable from their obvious similarity to hydropic degeneration in human diabetes, but the less advanced changes which lead up to them are almost more important, for they appear to correspond step by step with the progress of the disease. For instance, a dog is deprived of nearly nine-tenths of its pancreas and becomes glycosuric on a meat diet, but if the progress of the disease is favorable, glucose may disappear from the urine after a few days, only to return upon the administration of carbohydrate food. From this condition the dog may again recover and tolerate a moderate mixed diet without glycosuria. If such an animal is operated upon immediately, a fragment of the remaining portion of its pancreas will show in an occasional island the appearances illustrated by Figure 4. The majority of the cells of such islands are swollen, their outlines are distinct, their nuclei are prominent, their mitochondrial filaments are numerous, and their typical granules of secretion are rarely to be seen. Here evidently is the result of violent activity. The cells are perhaps healthy, but their granules are exhausted, and beside them are other cells whose appearance is not strikingly dissimilar, but whose protoplasm is vacuolated and nuclei shrunken. The latter are undergoing hydropic degeneration. Perhaps only a few islands of this sort may be found after a search through many normal sections, or, indeed, a few hydropically degenerate cells in one or two islands may be the only evidence of an abnormal strain upon the secretory mechanism. In this respect the dog appears to differ from the cat. In the latter, under the same conditions, there is no evidence of degeneration, but a uniform loss of "B" granules in all the cells of many islands, and with the loss of granules there is a prominence of mitochondria and nuclei suggestive of active metabolism. In the more advanced stages of experimental diabetes, however, appearances in both animals are similar.

Regenerative changes. — The alterations hitherto described in mild or violent experimental diabetes furnish evidence in the one case of over-activity, and in the other, of hydropic

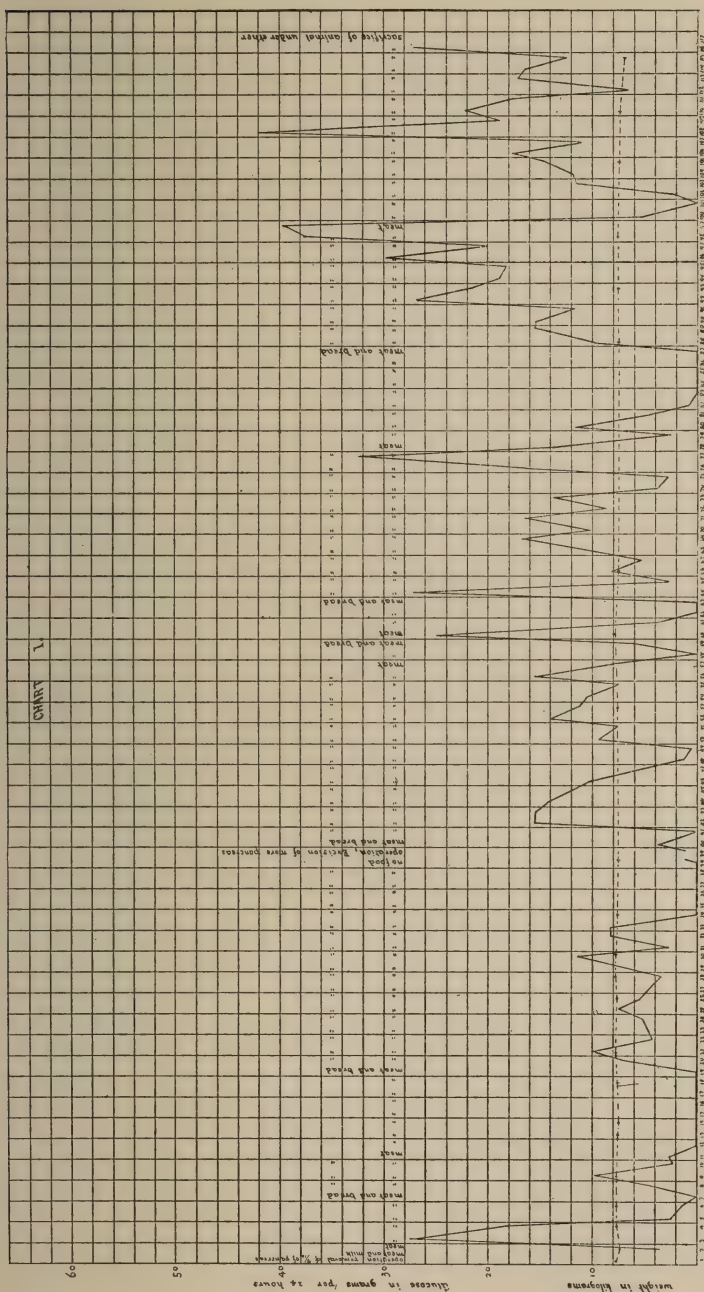
degeneration of the islands of Langerhans. Of regenerative changes, however, there is some evidence. Allusion has already been made to the animal which showed a tendency to recover from a rather severe diabetes "gravis," and in whom certain islands were seen which differed in several respects from the majority. These were small, better preserved than the rest, and contained no "A" cells. It was possible to follow them through serial sections so that there was no doubt as to the uniformity of their structure. Figure 8 gives a fair idea of their appearance, which may be compared with Figures 5 and 6, drawn from the islands in the same section. The better preservation of the cells in the former, the absence of "A" cells (upon which there is no apparent functional demand, for they never show any alterations), and the small size of the islands, suggest that they are new growths, probably from potential island tissue, in the ducts. The evidence is only suggestive, however, and demands further investigation.

From a review of the progressive changes in the islands in response to the removal of all but a small portion of the pancreas, one is led to the conclusion that the earlier changes are the result of excessive activity on the part of the island cells in response to a functional demand; that, as the inability of the body to utilize dextrose increases, the changes become more clearly degenerative; and that, when hydropic degeneration is fully established, the diabetic syndrome is complete and the body rapidly deteriorates. Experiments under the two following heads seem to offer an explanation of these phenomena.

2. Effect of carbohydrate food.—Experiments demonstrating the physical effect upon the islands of Langerhans of administering carbohydrate food to an animal in whom the greater part of the pancreas has been removed, with the conversion of a mild glycosuria into permanent experimental diabetes.

A typical experiment of this group will serve as an illustration (Observation IX., Chart I. A full account of this experiment will be found in the

Appendix. Similar experiments have been reported by Allen ²²). From a healthy young female dog an amount of pancreas estimated at about the usual nine-tenths was removed. A light transitory glycosuria upon meat diet followed. Bread was now added to the diet, but again the animal recovered from the ensuing glycosuria, and a second operation became necessary. About a fifth of the remaining fragment was excised and the dog was allowed bread and meat as before. For the next few weeks there followed glycosuria of moderate severity with but little polyuria, and a slight gain in weight. Immediately upon withdrawing bread from the diet, glycosuria ceased, returning at once upon the readministration of carbohydrate food, only to be promptly abolished upon the resumption of a pure meat diet. For the next two weeks a diet containing an abundance of bread was maintained. Glycosuria became intense, polyuria was noticeable, and when bread was again withdrawn, six days elapsed before sugar disappeared from the urine. The glycosuria which again followed the resumption of a mixed diet was the most severe which the animal had as yet experienced. The output of urine was large and the weight began to fall off. A genuine diabetes seemed to have become established (ninety-eight days after the beginning of the experiment and sixty days after the second operation). Two days after the final withdrawal of bread the urine was again free from glucose, but this time glycosuria returned with ever-increasing severity, even upon meat alone, and persisted at a high percentage until the end of the experiment. The animal, though bright and lively, was sacrificed two weeks after the definite establishment of diabetes.



OBSERVATION II (See Text and Appendix)
Effect of feeding Carbohydrates.

Drop of experimental observation
The values are collected in the A.M. Food given in the P.M.
The values for one day corresponds to first given the day before

Three sets of pancreatic tissue in this instance are available for microscopic study: the first, removed at the original operation, containing only resting islands loaded with granules; the second, taken from the pancreas just after the animal had succeeded in freeing itself from glycosuria on a mixed diet; and the third, removed after the sacrifice of the animal in the full tide of a violent diabetes. In the first specimen the islands are histologically normal, and it serves as a control for the second and third. In the second, many of the island cells are large and distinctly outlined, their nuclei and mitochondria prominent, and the "B" granules are considerably diminished in number. Many cells show evidence of early hydropic degeneration. Here, then, the islands have every appearance of physiologic activity with little degeneration, while the third set of specimens, obtained at autopsy, exhibit the classic picture of an advanced degenerative process.

One can hardly doubt that the element which has brought about this series of changes is the administration of carbohydrate food, and that, in the progressive loss of the animal's ability to utilize this food, lies an intimate resemblance to human diabetes. Moreover, the gradual lowering of the tolerance for carbohydrate corresponds apparently to progressive physical alterations in the islands. The steps of the changes ending in hydropic degeneration may perhaps be characterized as activity, exhaustion, and degeneration. Activity is here perhaps arbitrarily interpreted as a partial loss of the storage granules of the resting cell, accompanied by accentuation of the nucleus and mitochondria. It is seen in circumstances under which the organism is barely able to conquer a mild diabetes (Figures 3 and 4). Exhaustion is taken to be a stage of activity prolonged beyond normal physiological limits. Considerable swelling of cells, exhaustion of their granules, slight irregularity and shrinking of the nuclei, fusion of mitochondria into droplets, and slight vacuolization, characterizes this condition (Figures 3 and 4). Finally, in hydropic degeneration, which appears to follow exhaustion, the island cells are enormously swollen, their

nuclei pyknotic, and their protoplasm reduced to a few shreds. This change appears to be due to the absorption of fluid by the degenerate cell, the outlines of which may ultimately collapse and disappear (Figures 5, 6, and 7). It is strongly suggested, therefore, that activity, exhaustion, and degeneration are successively induced when the amount of island tissue is insufficient to meet a physiologic demand by the organism, and that the administration of carbohydrate food, by intensifying this demand, is a most potent influence in bringing about these histologic changes. The next series of experiments determines whether the assimilation of food, or the mere presence of food in the intestine, calls for this reaction.

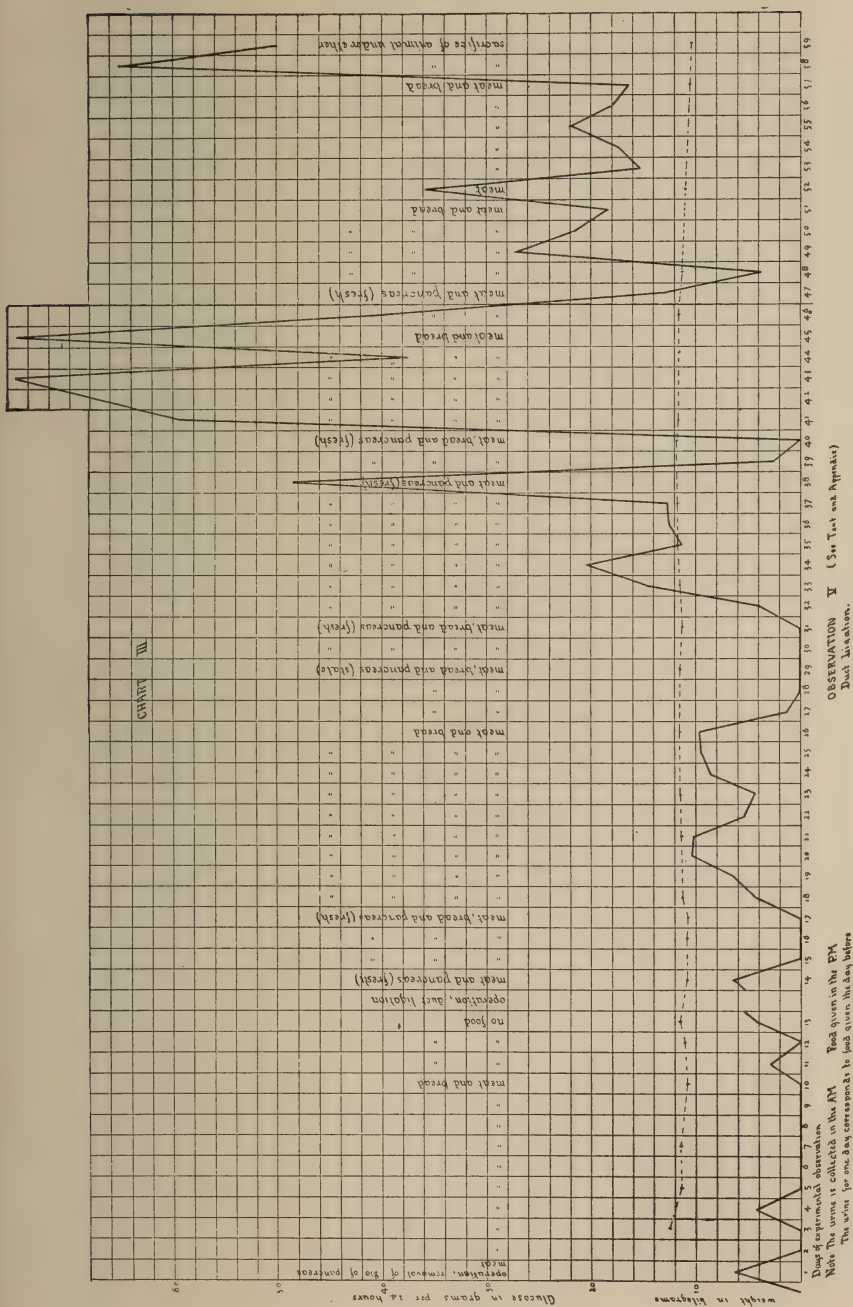
3. The effect of duct ligation.—These experiments demonstrate the physical effect upon the islands of Langerhans of duct ligation and the dietetic administration of fresh pancreas in the case of an animal with a much-reduced quantity of pancreatic tissue.

The success of the experiments cited under this head depends upon well-known facts already established by experiment: first, that exclusion of pancreatic juice from the intestine prevents the absorption of a considerable proportion of the food; and second, that ligation of the pancreatic duct (or ducts) brings about a slow atrophy, chiefly involving the acinous tissue, but not producing its full effects for several months.

Of the two successful experiments of this group, one (Observation V.) is sufficiently illustrative. A healthy young male Boston terrier was subjected to the usual removal of about nine-tenths of the pancreas, the remaining portion surrounding the chief duct. The result was the establishment of a glycosuria of moderate degree upon a diet of meat and bread, the diabetes "levis" of Allen, but instead of further reducing the bulk of the organ, the duct was divided between two silk ligatures, and, for additional security, a strip of omentum was inserted between the cut ends. At the same time a fragment of the gland was excised for microscopic study and a few islands exhibited the usual evidences of over-activity (Figure 4). Fresh pig's pancreas was now fed with meat. The appearance of the animal's movements indicated that the food had been

well digested and no glycosuria appeared until, some days later, bread was added to this diet. From this it appeared that after duct ligation, carbohydrate metabolism upon a meat, bread, and pancreas diet was equivalent to that upon meat and bread alone before the exclusion of pancreatic juice from the intestine.

From this time on, various changes in diet were instituted which brought out the following facts: On bread, meat, and pancreas, the animal gained weight and the food was well digested, but glycosuria steadily increased in intensity. Stale pancreas failed to give the same effect as fresh, showing that the destruction of the pancreatic ferments nullified the digestive action of the gland. On meat and bread alone glycosuria ceased, but the food was ill digested and the animal lost weight. Three weeks after duct ligation a diet of meat, bread, and pancreas was given without interruption, and the output of sugar in the urine rose to seventy-five grams in twenty-four hours (Chart III.). At this time (one month after duct ligation) the animal was fat and active, but upon withdrawing pancreas from the diet, it was found that permanent diabetes had become established, for alterations in the diet served only to alter the violence of the glycosuria. The dog then lost two kilograms in sixteen days and was sacrificed forty-seven days after ligation of the duct. Post-mortem examination disclosed a moderate atrophy of the acinous tissue and the usual advanced hydropic degeneration of the islands of Langerhans (Figure 7). (The accompanying Chart III. illustrates the effect of duct ligation and the administration of fresh pancreas upon the sugar output. In the Appendix will be found a full account of this experiment. Chart II. illustrates a similar experiment, Observation III., cited in detail in the Appendix.)



The feature of this experiment is the glycosuric effect of feeding fresh pancreas to an animal whose ability to assimilate nourishment is lowered by exclusion of the pancreatic juice from the intestine. Before duct ligation the dog became glycosuric upon a meat and bread diet. After ligation it was necessary to combine with carbohydrate feeding the administration of fresh pancreas to produce a similar effect. The most logical inference possible is that the fed pancreas brought on glycosuria by its digestive action in favoring the more complete assimilation of food. The experiment, therefore, furnishes evidence, confirming previous observations, that the assimilation of carbohydrate food in the presence of an already lowered carbohydrate tolerance has a destructive effect upon the dog's pancreas, and shows that the mere presence of this food in the intestine is of no influence in this respect. Incidentally, it disposes of the possibility that a diabetic animal may be benefited by the administration of fresh pancreas.

4. The effect of nervous stimuli.—The experiments which follow are intended to test the effect of certain nervous stimuli upon glycosuria and upon the physical character of the islands of Langerhans.

The observations already cited bring evidence connecting the internal secretion of the islands of Langerhans with the utilization of dextrose, for they demonstrate that removal of a large proportion of the total quantity of island tissue lays the remainder open to a deteriorating influence, which is intensified by the addition of carbohydrates to the diet. But they furnish no evidence not otherwise available that the islands have a predominating glycogenic or glycolytic function. It seems advisable, therefore, to supply to the tissues an excessive amount of dextrose in the form in which they are accustomed to use it, and to determine whether, as the amount of pancreas is reduced, there is a waste of this material, that is, a failure of glycolysis. There are two easily available ways of measuring the demand of this excess of dextrose upon the internal secretion of the islands: first,

the relative amount of glucose excreted in the urine when the pancreas is intact, or reduced by an amount nearly sufficient to allow spontaneous glycosuria; and second, the appearance of the islands themselves under these conditions. The introduction of an excess of dextrose into the circulation presents little difficulty, but in order to make certain that it is in a form in which the tissues are normally able to consume it, a method of discharging it from liver glycogen, its natural source, must be devised. This means is furnished by stimulation of the splanchnic nerves.

A number of important observations have a bearing upon this physiological reaction. Electrical stimulation of the splanchnic nerves is known to cause a discharge of adrenalin, accompanied by hyperglycemia and glycosuria. MacLeod's²³ experiments indicate that the mechanism by which liver glycogen is broken down into dextrose resides in the sympathetic nervous system, and that the integrity of the adrenals is necessary to its successful action. Elliot²⁴ finds that the adrenal medulla is discharged with glycosuric effect by many reflex influences if the splanchnic nerves are intact. Recently, Cannon²⁵ has demonstrated that emotional glycosuria and hyperglycemia are brought about by excitation of this same mechanism. He suggests that the emotional liberation of adrenalin and dextrose has a useful purpose, namely, to afford a supply of nourishment for sudden intense effort on the part of the muscles, and this suggestion derives support in the observations of Lusk and Riche,²⁶ that the presence of adrenalin in the blood is not unfavorable to the consumption of dextrose by the body.

It appears, then, that electrical excitation of the splanchnic nerves is at once an adequate and natural means of supplying dextrose to the tissues in testing the glycolytic power of the pancreas. In order, however, to obtain information of any reliability as to the effect of splanchnic excitation, it seems probable that the stimulation must be maintained for many hours. In the earlier experiments, owing to certain technical difficulties, the secretion of urine ceased after a few hours, the temperature fell and the animal died; but later it

was possible to keep an animal in good condition and to maintain, for the observation of glycosuria, a flow of urine for seven to ten hours.

The technical procedure finally adopted is as follows: The dog is anesthetized lightly with ether and placed on his back on a warm pad. The abdomen is opened for the excision of a small fragment of pancreas, which is immediately placed in granule fixatives as a control to subsequent changes. Through a second small opening in the left flank, a glass tube connected with a warm water tank is sewed into the descending colon as a means of supplying an abundance of fluid to the animal during its long anesthesia. Urine is collected through a catheter.

Intratracheal insufflation now takes the place of the cone anesthesia, and the animal is kept during the rest of the experiment so lightly anesthetized that superficial reflexes are always active. Openings are made into the chest through the tenth interspaces, the spinal roots to the splanchnics divided from the main trunk below the point to which stimulation is to be applied, and above the splanchnic nerves freed from their beds and cut above. A hooked bipolar electrode embedded in a pointed wooden handle, which can be thrust into the muscles of the chest wall close to the spine, is applied to each nerve, and the chest is closed hermetically about the projecting handles. This whole procedure takes about an hour. As soon as the first splanchnic is ready, stimulation by a faradic current just sufficient to contract exposed muscle is begun, and when the second electrode is applied the current is turned alternately into each nerve for two or three periods of five seconds, every ten, and sometimes every five minutes. During the experiment the flow of urine is fairly constant. No sugar appears, as a usual thing, until after handling of the splanchnics is begun, and the flow of urine continues for from seven to ten hours, when the pulse begins to become small and feeble. The experiment is brought to an end by deepening the anesthesia and opening both sides of the chest.

Under the conditions described above the splanchnics have been stimulated: *First*, in animals with an intact pancreas; *second*, in others, with the pancreas cut down about one-half; and *finally*, in a third group, after removal of about four-fifths of the organ — that is, nearly enough to bring about spontaneous glycosuria.

In the first series, carried out without removal of any of the pancreas, it was established that the mere administration of ether did not induce glycosuria, but that handling and division of the splanchnics might excite it even before faradization was begun. In the earlier experiments a glycosuria

of four per cent was the highest observed. Stimulation was maintained in one instance for seven hours. Sugar appeared in the urine about one hour after division of one splanchnic nerve, and fifty minutes after the first faradization. The amount increased in two hours to 1.8 per cent and declined from this time to one per cent at the termination of the experiment, when eighty-four cubic centimeters of urine had been secreted. That the discharge of adrenalin was vigorous is affirmed by the fact that before death the femoral artery, owing to the contraction of its walls, spurted only a very fine stream on section, and that the blood clotted almost instantly. A comparison of the control specimen of pancreas (removed before stimulation) with the autopsy specimen discloses in the latter a slight increase in activity in the island cells, witnessed by swelling and a moderate loss of storage granules in a few cells—not a striking change, if indeed it can be asserted that it is outside the limit of error in fixation and staining. As the abnormalities in organs other than the pancreas are practically constant through the series, a description of this case will cover all subsequent experiments. The intestines are pale, the great abdominal veins are engorged, the spleen is large, dark red, and bloody on section. The adrenals do not appear remarkable, though the medulla seems narrower and firmer than usual.

In the second series two experiments were performed after removal of about one-half of the pancreas. In the first of these, twenty-nine days elapsed after resection, and in the meantime the animal had gained weight from 13.5 to 15.5 kilograms, and tolerated a meat and bread diet without glycosuria. Stimulation was maintained for nine hours. Sugar appeared in the urine after handling, division, and the first faradization of the splanchnic nerves, or possibly even during handling. Glycosuria originally of four per cent fell to 1.5 per cent in two hours and from then on never below 1.2 per cent. The total quantity of urine was 91.5 cubic centimeters. The usual evidences of intense adrenal discharge were found post-mortem, but no difference could be

detected between the specimens of pancreas removed at the beginning and end of splanchnic stimulation.

The second experiment of this group differs from the first in that, owing to an escape of pancreatic juice from the divided end of the remaining portion of the pancreas, a cyst formed which partly obstructed the pylorus. The animal ate only a small amount of meat, and in the thirteen days which elapsed between resection of the pancreas and the final experiment lost two kilograms in weight. Sugar appeared in the urine promptly after stimulation, but the percentage never rose above .8 per cent and fell to less than .5 per cent before the end of the experiment. Faradization of the splanchnic nerves was maintained for ten hours and caused such an active discharge of adrenalin that neither the carotid nor the femoral artery, when divided, emitted more than a fine spurt, while the spleen, the liver, and the portal vessels were unusually engorged. In the pancreas no change beyond slight evidences of island activity is observable. Moreover, from a comparison of the sugar output in these two experiments, it appears that the intensity of glycosuria depends more upon the condition of nourishment of the animal (available glycogen) than upon the amount of pancreas removed.

In the third series one experiment was performed after removal of four-fifths to five-sixths of the pancreas. The animal, in the nine days which intervened between resection of the pancreas and the final experiment, was able to take a bread and meat diet without glycosuria, and lost only half a kilogram of weight. No sugar appeared in the urine as a result of the operative procedure until after faradization of the splanchnics, and the percentage never rose above 1.2 per cent in a total urine output of 82.6 cubic centimeters. Stimulation was maintained for nine hours. Of evidence of increased island activity or of degeneration following splanchnic stimulation, there is absolutely none in the autopsy specimens of the pancreas.

Control experiment.—The only observation which can possibly be called a control upon these procedures was performed upon an animal

which, after removal of about four-fifths of the pancreas, suffered at first from glycosuria on a meat diet. Upon reduction of the amount of meat, however, the glycosuria ceased and the dog was able at the time of the final experiment to tolerate even a small amount of carbohydrate food. The procedure in this instance was varied in no respect except that the splanchnics were cut, but not faradized. Sugar in small amount appeared as a result of anesthesia alone, and division of the splanchnics was followed by a glycosuria of 3.3 per cent. After this, the course of the experiment was very different from that of all former observations. The output of urine fell rapidly to nothing during the first hour, and the percentage of sugar in the last urine secreted at the end of this time was only 2.5 per cent. Evidently the interruption of all sympathetic impulses to the abdominal viscera caused a marked fall of blood pressure, for, accompanying the cessation of renal activity, the pulse gradually became softer and the heart stopped beating four hours after splanchnic division.

The post-mortem appearances, as might have been expected, are quite different from those of previous experiments. The venous engorgement of the spleen and large abdominal veins is absent and the adrenal medulla appears thicker, softer, and more friable. In the pancreas, however, the islands, though exhibiting alterations obviously suggestive of over-activity, are quite alike in the control and the post-mortem specimens. There is evidence, in the altered appearance of the islands, of the recent glycosuria from which the animal had suffered in consequence of pancreatic resection, but no sign of further alterations following the final experiment. This observation shows that excitation by ether (probably a sympathetic affair), before division of the splanchnics, is capable of causing glycosuria in an animal very close to the border of carbohydrate intolerance—a glycosuria which is temporarily intensified by handling and division of the splanchnic nerves. It appears, therefore, that when the loss of island secretion is carried to a point where it is barely sufficient to hold off glycosuria, the glycogenolytic action of the sympathetic system is more rapidly effective.

One would be justified, then, in drawing from these three groups of observations the following conclusions: first, that electrical excitation of the splanchnic nerves causes a discharge of adrenalin into the blood and the excretion of

sugar by the urine; second, that ether alone may rapidly induce glycosuria when the amount of pancreas present in the body is otherwise barely sufficient to keep the urine sugar free; third, that the *intensity* of glycosuria resulting from splanchnic stimulation under these conditions is not at all influenced by the amount of pancreas present in the body, but rather by the animal's available supply of glycogen; and, finally, that there is no reason at present to believe that electrical splanchnic stimulation, or a temporary excess of adrenalin in the blood, or an excess of sugar in the blood, or, in fact, that the glycogenolytic action brought about by faradization of the sympathetic system, calls for any noticeable activity on the part of the islands of Langerhans. This conclusion must be accepted without prejudice to the possible effect of some more prolonged physiological excitation, for it may appear that by its action in breaking down excessive amounts of glycogen into dextrose, the sympathetic mechanism may in time impose a call indirectly upon glycogenic activities in the islands, similar in effect to that imposed by over-feeding with carbohydrates an animal whose islands are much reduced in number. There is nothing in evidence, however, at the present time to support this hypothesis.

DISCUSSION OF EXPERIMENTAL DIABETES AND ITS RELATION TO DIABETES IN MAN.

The relation of diet to alterations in the islands of Langerhans in experimental diabetes.—The importance of the observations which form the basis of this study depends in great measure upon the interpretation placed upon the physical changes, already described, in the islands of Langerhans. These changes appear when an animal is deprived of a large part of its pancreas. They may appear, under these circumstances, when the animal is subsisting entirely upon meat. They may appear upon the assimilation of carbohydrates, after a meat diet has failed to produce them. The assimilation of food, and more especially carbohydrate

food, is, therefore, closely related to changes in the cells of the islands.

These alterations in the islands of Langerhans have already, upon the evidence of microscopic study, been shown to betoken *activity, exhaustion, and degeneration*. The changes are consecutive in any one experimental observation. They accompany and keep pace with a progressive failure on the part of the individual to make use of dextrose; in other words, their progression parallels a diabetic tendency. It has, therefore, been assumed that activity in island cells, in association with evidence (glycosuria) of an approaching diabetic state, induced by the removal of an appropriate amount of pancreas, is a response to a call upon a normal physiological function of the islands; and that the eventual exhaustion and degeneration in fully-developed experimental diabetes, is the result of over-activity in response to a prolonged demand. Such an assumption appears to be warranted by the well-recognized dependence of carbohydrate metabolism upon the secretion of the islands.

Yet it may be objected that a physiological call upon a secreting gland — a stimulating mechanism — has never been known to bring about the destruction of the gland. To this objection it may be replied that there is perhaps no other ductless gland in the body, save perhaps the adrenal medulla, which is known to be subject to such insistent demands upon its activity, and in which over-stimulation has been studied by appropriate methods. Moreover, it is known that the adrenal medulla, possibly the whole gland, may, in consequence of Bernard's piqûre, become exhausted, with loss of its granules of secretion, general vacuolization, and swelling of its cells (Kahn²⁷); and it has, likewise, been established by experiment that violent over-stimulation by "secretin" (Bayliss and Starling²⁸) completely discharges the zymogen of the pancreatic acinous tissue with the production of considerable vacuolization. (The writer obtained this result in experiments performed under Professor Starling's direction in 1912. A drawing made from an "exhausted" pancreas at this time shows how profoundly the acinous

tissue is affected.²⁹⁾ It has never been proved that it is possible to destroy either of these tissues by such temporary over-stimulation, yet exhaustion by the unnatural prolongation of a normal stimulus, though carried only to a point from which recovery is possible, offers an analogy to permanent exhaustion of the islands of Langerhans in experimental diabetes.

A further explanation of the degenerative effect upon the islands of the assimilation of food after nearly complete pancreas extirpation is suggested by the answer to another question: Are degenerative alterations an effect or a cause of experimental diabetes? They are, in fact, a cause only, for though the islands of the small fragment show evidence, first of over-activity, and finally of degeneration in responding to a demand for their secretion, the changes which occur in them are an effect of a normal stimulation. Only by their failure to respond — the result of exhaustion — can a diabetic condition become established. In this connection it will be remembered that the islands of an animal which has recovered from a moderately-severe glycosuria upon a meat diet appear for the most part normal (Appendix, Obs. V. Figure 4), while those of the same animal afterward rendered fatally diabetic by prolonged carbohydrate feeding beyond the limit of tolerance are hopelessly degenerate, and this soon after the disease has become established.

The relation of the lesions of experimental diabetes to those of diabetes in man. — The demonstration by Allen and the writer of "hydropic" degeneration as a widespread and easily-recognized lesion in experimental diabetes explains and develops the previous discovery by Weichselbaum of a similar form of degeneration in the diabetes of man. (In Allen's book, "Glycosuria and Diabetes," may be found excellent photomicrographs of islands undergoing "hydropic" degeneration.) Moreover, the recognition in animals of the steps leading up to this degeneration makes clear the failure of so many investigators to discover evidence of

disease in the diabetic human pancreas. For it is not until one is familiar with these progressive changes that one can recognize them in anything short of their violent terminal form. A drawing (Figure 9) made from a diabetic human pancreas is submitted to illustrate this point. The tissues were obtained within an hour of death and preserved in the same fixatives used to demonstrate secretory granules in animals. The fixation is not perfect, but the preservation of zymogen in the acinous cells, and the presence of typical granules in many of the cells of the island, vouch for the absence of post-mortem changes. In one corner of a large island are a number of typically exhausted and several hydropically degenerate cells, and though the number of stricken cells is perhaps no greater than is commonly seen in mild diabetes of the dog, the similarity between the experimental and the natural diabetes is rendered all the more interesting in that the human subject had suffered from diabetes for only two years and died from intercurrent disease. Such a slight change would not have been recognizable in tissues exposed to more than a few hours of post-mortem decomposition, for all secretory granules rapidly disappear after death. It is, therefore, only the very advanced form of the degeneration which is easily recognized under these conditions, and the wonder is that Weichselbaum is able to find evidence of it in fifty per cent of one hundred and eighty-three autopsies, more especially when it is realized that many diabetics die, not in the terminal stage of diabetes, but of intercurrent maladies.

For the more chronic alterations described by Opie, Heiberg,³⁰ Cecil,³¹ and others, experimental diabetes offers no support, unless one admits, as is quite reasonable, that hyaline, fibrous or fatty deposits, may obliterate the traces of earlier hydropic degeneration of the island cells. No such process, however, has been observed in the comparatively rapid diabetes of animals. One may, then, accept this possibility, or hold that hyaline and fibrous degeneration may, themselves, occur in response to the same influences which bring about hydropic degeneration, and so destroy

the islands, or unless one is prepared to argue that chronic degenerative changes of an accidental character may destroy a high proportion of the islands, one had better cease to regard such changes as of importance in the pathology of diabetes.

The relation of the clinical manifestations of experimental diabetes to diabetes in man. — The clinical manifestations, like the pathological changes of the experimental disease, offer a close analogy to human diabetes and open wide fields of investigation. Experiments dealing with the results of feeding carbohydrates beyond the limit of tolerance, both with and without the exclusion of pancreatic juice from the intestine, bring fresh evidence of the intimate relation of the glycogenic function of the pancreas to human diabetes. If the assimilation of carbohydrate passes the point of the organism's ability to deal with it, the islands of Langerhans exhibit evidence of overstrain with overflow of dextrose into the urine. If the assimilation of carbohydrate is checked by exclusion of this food from the diet or by exclusion of the pancreatic juice from the intestine (which amounts to the same thing), the glycosuria ceases and the islands of Langerhans remain in a state of health. If the feeding of carbohydrate is long continued the islands again feel the strain of governing, if one may say so, the assimilation of dextrose. Degeneration, which invariably follows a long continuance of this strain, is accompanied by the cessation of the animal's ability to make any use whatever of dextrose. Thus a functional is converted into an organic disease.

Allen has very ingeniously taken advantage of the knowledge derived from experimental research of this kind. He discovered in animals that if, after the establishment of what appeared to be a fatal experimental diabetes, food were withheld, glycosuria ceased, and that he could then feel upward, so to speak, for the low limit of the quantity of food which the animal could tolerate without glycosuria. Having made this determination, he was able to keep an animal

sugar free at a low metabolic level, though it never could be made to gain weight without a return of the diabetic syndrome. He has applied this absolutely sound principle to human patients with a considerable degree of success at the Rockefeller Hospital in New York.³³

The function of the island secretion probably glycogenic. — It must appear clear to those who accept these experimental observations that the pancreas is concerned principally with the assimilation and storage of dextrose, a conclusion entirely in agreement with what was previously known of its function. Yet in diabetes of man, as in experimental diabetes of animals, there is obviously a disturbance of the power of consuming (glycolysis) as well as of building up (glycogenesis) dextrose. The experiments concerning the relation of the islands of Langerhans to the mechanism which liberates dextrose for body use are attempts to throw light upon the function of the pancreas in glycolysis. If the islands have a glycolytic function, it seems probable that they will show evidence of activity when a quantity of dextrose is suddenly broken down from the liver glycogen. The result of the fourth group of experiments, though not entirely convincing, is at least suggestive that the activities of the islands are not directly related to a glycolytic mechanism.

One may, then, fall back upon any of several explanations of the failure of glycolysis in diabetes. In the light of this research it is a plausible assumption that the disturbance of the glycogenic function of the pancreas so alters the chemistry of the carbohydrates supplied to the body that the tissues are unable to consume them in the form in which they appear in the circulation. In support of this hypothesis a very plausible line of reasoning may be invoked. The pancreas is placed in the portal circulation. The products of its metabolism, if, as is probable, they enter the portal veins, go entirely to the liver. Both the external and internal secretion of the pancreas are concerned with digestion, — the first in a narrower sense, with the preparation of

food for transmission through the intestinal wall, and the latter in a broader sense, with preparation of the food for the use of the body. The island secretion is certainly of vital importance to the storage of carbohydrate as glycogen in the liver, for in experimental diabetes the liver is found to be glycogen free (examination by glycogen fixatives and stains — in protocols in Appendix). When, therefore, owing to the failure of the internal secretion of the pancreas, carbohydrate material (dextrose) is not first fixed as glycogen in the liver for subsequent distribution to the body, but passes through the liver into the general circulation, it is incompletely burned. That the dextrose of diabetics is offered to the tissues in a form in which they are unable to consume it is an attractive hypothesis, as more than one investigator has hinted. Whether this hypothesis should be accepted or whether the fundamental cause of the failure of the diabetic tissues to consume dextrose lies in the tissues themselves, or in some direct relation between them and the internal secretion of the pancreas, are problems still to be solved.

RECAPITULATION OF THE RELATIONS BETWEEN HUMAN AND EXPERIMENTAL DIABETES.

1. Pathological relations. — A basis for the comparison of experimental and human diabetes has already been indicated; an important similarity between the pathologic changes found in each disease has been described; and an explanation of the discrepancies between the changes in the one and the other suggested. Should it prove possible to study the human diabetic pancreas by histologic methods now available in the case of the experimental animal, the same downward steps, beginning in activity and ending in hydropic degeneration, may well be found in the islands of Langerhans.

2. Etiologic relations. — For the etiology of human diabetes, the experimental disease offers a less satisfactory

explanation. A reduction of the dog's pancreas to a rough tenth of its normal size brings about so great a deficiency of island tissue that the remainder is insufficient to meet the demands of carbohydrate metabolism. Some influence associated with the utilization of carbohydrates — an influence whose nature is not understood — then calls upon the remaining islands for unusual secretory efforts. The failure of these islands adequately to functionate in response to this influence is the basis of experimental diabetes. Human diabetes, if its clinical resemblance to the experimental disease is not meaningless, takes origin in a similar failure of island secretion; but whether, in the intact human pancreas, the islands break down in response to a too-often repeated normal stimulus, or whether the diabetic islands are themselves functionally weak is still to be determined.

Considering the pancreas as one organ, with two allied functions, the thought is stimulating that the islands may be activated through the blood stream by a mechanism analogous to the activation of the acini by the "secretin" of the intestinal mucous membrane. It is indeed to be hoped that the uncertainty which still hangs over the etiology of human diabetes will ultimately be cleared away by some such discovery as is here suggested.

3. Clinical relations. — The progress of experimental diabetes in the canine, though extremely rapid in comparison with the chronic course of diabetes in man, resembles it in many respects. The administration of carbohydrate food intensifies the waste of dextrose and hastens the progress of both diseases. In both, the body becomes emaciated by burning as many of its own tissues as are available. In both, there is a tendency to a fatal outcome. The life of a human diabetic, however, is well known to be lengthened if the individual is kept free, by dietary measures, from carbohydrate waste (glycosuria). A similar observation is suggested by the experimental observations of the writer, and has been actually demonstrated by Allen, and applied by him with encouraging evidence of success in man.³³

Experimental and human diabetes are, therefore, strikingly alike. The experimental disease is due to a functional deficiency on the part of the islands of Langerhans. Diabetes of man has in all probability a similar immediate etiology.

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APPENDIX.

I. Experiments demonstrating various stages of functional activity and degeneration in the islands of Langerhans:

OBSERVATION VIII. — Summary: Young, full-grown, male mongrel. Weight 11.16 kilograms. Removal of nine-tenths of pancreas. Permanent diabetes. Intercurrent distemper. Sacrifice of animal when emaciated, twenty-seven days after operation.

2-19-14.	Urine 120 cc.	S.G. 1.025.	Sugar 0.		
2-20-14.	Operation.	Removal of all but a small portion of the pancreas immediately surrounding the opening of the principal duct into the intestine. Weight of pancreas removed 22 grams, estimated at about nine-tenths of the whole. A dry, rapid operation. No food was given on the day of operation.			
2-21-14.	No urine passed.	Diet * meat and milk.	Wt. 10.88 kilos.		
2-22-14.	Urine 300 cc.	Sugar 2.5%.	Meat.	Wt. 10.86 kilos.	
2-24-14.	" (48 hrs.)	750 cc. Sugar 6.4%.	Meat.	Wt. 10 kilos.	Cough and nasal discharge.
2-25-14.	"	370 cc. Sugar 8.0%.	Meat.	Wt. 9.8 kilos.	Nasal discharge, but does not seem sick.
2-26-14.	"	150 "	" 5.7%.	"	Mild distemper.
2-27-14.	"	400 "	" 5.5%.	"	Distemper better.
2-28-14.	"	700 "	" 3.6%.	"	Wt. 9.5 kilos. Weak.
3-1-14.	"	350 "	" 4.3%.	"	" 9.2 " Responsive.
3-2-14.	"	600 "	" 3.3%.	"	" 9.26 " Diarrhea.
3-3-14.	"	450 "	" 4.0%.	"	" 9.0 " "
3-4-14.	"	410 "	" 3.3%.	"	" 8.96 " "
3-5-14.	"	500 "	" 3.4%.	"	" 8.66 " "
3-6-14.	"	300 "	" 6.1%.	Meat and milk.	"
3-7-14.	"	600 "	" 5.8%.	" " "	Wt. 8.76 kilos. Cough.
3-8-14.	"	450 "	" 3.2%.	" " "	" 8.5 " Less diarrhea.
3-9-14.	"	120 "	" 6.9%.	" " "	" 8.48 " No diarrhea.
3-10-14.	"	300 "	" 8.7%.	Meat.	Wt. 8.6 kilos. Nasal discharge.
3-11-14.	"	lost.	"	"	Diarrhea.
3-12-14.	"	410 "	" 6.6%.	"	Wt. 8.6 kilos. Better.
3-13-14.	"	380 "	" 7.0%.	"	" 8.6 " Nearly well.
3-14-14.	"	500 "	" 4.0%.	"	Diarrhea.
3-15-14.	"	200 "	" 5.0%.	"	" 8.16 " "
3-16-14.	"	250 "	" 4.0%.	"	No diarrhea.

* The relation of the diet to the collection of urine is as follows: the urine is collected each day before feeding; therefore the output of sugar in the urine for one day is influenced by the diet of the day before.

3-17-14.	Urine 270 cc.	Sugar 3.7%.	Meat and bread.	Wt. 7.92 kilos.	No diarrhea.
3-18-14.	" 830 "	" 6.6%.	" " "	" 7.7 kilos.	Slight diarrhea.
3-19-14.	" 550 "	" 6.1%.	Meat.	Wt. 7.88 kilos.	Slight "
3-20-14.	" 430 "	" 2.7%.	" 7.4 "	No "	"

The animal was now very weak, and had lost about 3.5 kilograms since the beginning of the experiment. Though distemper was better it was not cured, and it seemed best to sacrifice the animal.

Animal sacrificed by bleeding under ether.

Autopsy: Gross appearances not remarkable except that the fragment of pancreas was greatly hypertrophied. Its duct was patent. Its weight was 6 grams, or about three times its estimated weight. Microscopic examination: Pancreas, acinous tissue normal; islands of Langerhans in advanced stage of hydropic degeneration (Figures 5 and 6); some islands show evidence of regeneration (Figure 8). Liver and kidneys, not remarkable; almost no glycogen. Adrenals, not remarkable.

OBSERVATION XI.—Summary: Middle-aged, male, mongrel bull terrier. Weight 11.6 kilograms. Removal of about nine-tenths of pancreas, a long but dry operation. No glycosuria on meat diet. Transient glycosuria (for one day) on meat and bread. Second operation. Hypertrophy of fragment, about one-third of which was removed. Intermittent glycosuria on meat diet, becoming more severe. Animal in extremis seven weeks after second operation, and was sacrificed.

Autopsy incomplete: Extreme hydropic degeneration of islands.

5-18-14.	Operation.	Removal of amount of pancreas estimated at nine-tenths.			
	No food this day.	Weight 11.6 kilos.			
5-19-14.	Urine 200 cc.	Sugar O.	Meat only.		
5-20-14.	" 230 "	" O.	" "		
5-21-14.	" 150 "	" O.	" "	Wt. 11 kilos.	
5-22-14.	" 420 "	" O.	" "	" 10.7 "	
5-23-14.	" 190 "	" O.	" "		
5-24-14 to 6-1-14.	No glycosuria.	Meat only.	Loss of weight to 10 kilos.		
6-2-14.	Urine O.	Meat and bread.	Wt. 9.94 kilos.		
6-3-14.	" 180 cc.	Sugar O.	Meat and bread.		
6-4-14.	" 250 "	" O.	" "	Wt. 10.3 kilos.	
6-5-14.	" 140 "	" 1.2%.	" "		
6-6-14 to 6-30-14.	No glycosuria.	" "	Wt. rose to 11.1 kilos.		
7-1-14.	Second operation.	Removal of one-third of the remains of the pancreas, which seems to be hypertrophied.	Examination of specimen disclosed normal islands with some evidence of over-activity but no degeneration.	No food this day.	

7-2-14.	Urine 120 cc.	Sugar 3.3%.	Meat only.	
7-3-14.	" 200 "	" O.	" "	Wt. 10.04 kilos.
7-4-14.	" 210 "	" O.	" "	Fine condition.
7-6-14.	" 630 "	" O.	" "	Wt. 10.4 kilos.
7-7-14.	" 400 "	" 1.5%.	" "	
7-8-14.	" 420 "	" 2.1%.	" "	
7-9-14.	" 150 "	" O.	" "	Wt. 10.3 kilos.
7-10-14.	" 210 "	" O.	" "	
7-12-14.	" 500 "	" 1.4%.	" "	
7-13-14.	" 110 "	" .9%.	" "	
7-14-14.	" 85 "	" O.	" "	Wt. 9.94 kilos.
7-16-14.	Animal running free. Given bread by mistake yesterday and to-day. No observation of urine. Meat from now on.			
7-28-14.	Urine 260 cc.	Sugar 3.8%.	To have meat from now on and to run free with other animals.	
8-21-14.	Has become much emaciated and very weak. Sacrificed by bleeding under ether.			

Autopsy incomplete: Pancreas shows extreme hydropic degeneration of the islands of Langerhans.

GROUP II.—Experiments demonstrating the physical effect upon the islands of Langerhans of administering carbohydrate food to an animal in whom the greater part of the pancreas has been removed, with the conversion of a mild glycosuria into permanent experimental diabetes.

OBSERVATION IX. (See Chart I. This observation is quoted in the text.) — Summary: Fox terrier. Female. Young adult. Weight 8 kilograms. Removal of nine-tenths of pancreas. Glycosuria on a meat diet. Recovery: Glycosuria on a meat and bread diet, followed by toleration of bread and meat without glycosuria. Second operation. Removal of more pancreas. Specimens show slight exhaustion of island granules. Subsequent glycosuria on meat and bread diet gradually converted into permanent, fatal diabetes by carbohydrate feeding. Sacrifice of animal 116 days after first operation and 18 days after final establishment of diabetes. Autopsy shows advanced hydropic degeneration of islands of Langerhans.

3-6-14.	Operation. Removal of an amount of pancreas estimated at nine-tenths. No food this day.			
3-7-14.	Urine O.	Meat and milk.	Wt. 7.86 kilos.	
3-8-14.	" 300 cc.	Sugar 1.2%.	Meat only.	
3-9-14.	" 420 "	" 6.6%.	" "	Wt. 7.8 kilos.
3-10-14.	" 300 "	" 6.1%.	" "	
3-11-14.	" 220 "	" 1.1%.	" "	
3-12-14.	" 160 "	" .88%.	" "	Wt. 7.66 kilos.

3-13-14.	Urine 160 cc.	Sugar O.	Meat and bread.	
3-14-14.	" 240 "	" 2.0%.	" " "	
3-15-14.	" 160 "	" 6.1%.	" " "	Wt. 7.66 kilos.
3-16-14.	" 130 "	" 1.8%.	" " "	
3-17-14.	" 140 "	" 2.0%.	Meat only.	Wt. 7.68 kilos.
3-18-14.	" 230 "	" O.	" "	
3-19-14 to 3-24-14.	No glycosuria.		" "	Wt. 7.6 kilos.
3-25-14.	Urine 160 cc.	Sugar O.	Meat and bread.	
3-27-14.	" 280 "	" 3.6%.	" " "	Wt. 7.8 kilos.
3-28-14.	" 220 "	" 3.6%.	" " "	
3-29-14.	" 540 "	" 3.3%.	" " "	
3-30-14.	" 280 "	" 1.9%.	" " "	Wt. 7.6 kilos.
3-31-14.	" 310 "	" 2.4%.	" " "	
4-1-14.	" 240 "	" 2.3%.	" " "	
4-2-14.	" 500 "	" O.	" " "	
4-3-14.	" 370 "	" 1.0%.	" " "	Wt. 7.8 kilos.
4-5-14.	" 550 "	" 2.1%.	" " "	
4-6-14.	" 470 "	" 0.6%.	Meat and much bread.	
4-7-14.	" 620 "	" 1.3%.	" " "	
4-8-14.	" 390 "	" 2.1%.	" " "	Wt. 7.6 kilos.
4-10-14 to 4-14-14.	No glycosuria.		Same diet.	Wt. 7.5 kilos.
4-14-14.	Operation. Removal of about one-quarter of fragment of pancreas. Specimen shows moderate exhaustion of granules in islands. No food this day.			
4-15-14.	Urine 150 cc.	Sugar 2.5%.	Meat and bread.	
4-16-14.	" 170 "	" O.	" " "	
4-17-14.	" 310 "	" 5.0%.	" " "	
4-18-14.	" 280 "	" 5.5%.	" " "	
4-19-14.	" 330 "	" 4.3%.	" " "	
4-21-14.	" 430 "	" 2.4%.	" " "	
4-27-14.	" 280 "	" 5.0%.	" " "	
4-28-14.	" 310 "	" 3.6%.	" " "	Wt. 7.7 kilos.
5-2-14.	" 180 "	" 4.4%.	Meat only.	
5-3-14.	" 210 "	" O.	" "	
5-4-14.	" 600 "	" 1.0%.	Meat and bread.	
5-5-14.	" 710 "	" 3.5%.	Meat only.	Wt. 6.94 kilos.
5-6-14.	" 300 "	" 1.25%.	" "	
5-7-14.	" 130 "	" O.	" "	
5-8-14.	" 230 "	" O.	Meat and bread.	
5-9-14.	" 540 "	" 5.0%.	" " "	
5-10-14.	" 140 "	" 2.0%.	" " "	
5-11-14.	" 300 "	" 2.7%.	" " "	Wt. 7.68 kilos.
5-12-14.	" 280 "	" 1.9%.	" " "	
5-14-14.	" 510 "	" 3.3%.	" " "	
5-15-14.	" 270 "	" 3.8%.	" " "	
5-16-14.	" 410 "	" 4.0%.	" " "	
5-22-14.	" 520 "	" 6.2%.	" " "	

5-23-14.	Urine 220 cc.	Sugar 6.0%	Meat only.	
5-24-14.	" 170 "	" 1.5%	" "	
5-25-14.	" 180 "	" 6.4%	" "	
5-26-14.	" 85 "	" 5.3%	" "	Wt. 7.86 kilos.
5-27-14.	" 81 "	" 1.0%	" "	
5-28-14.	" 220 "	" O.	" "	
5-29-14 to 6-1-14.	No glycosuria. Meat only. Bread and meat on 6-1-14.			
6-2-14.	Urine 170 cc.	Sugar 5.7%	Meat and bread.	Wt. 7.66 kilos.
6-3-14.	" 300 "	" 5.2%	" " "	
6-4-14 to 6-12-14.	Highest glycosuria. 7.3%. Bread and meat diet.			
6-13-14.	Urine 600 cc.	Sugar 6.6%	Meat only.	
6-14-14.	" 90 "	" 5.7%	" "	
6-15-14.	" 200 "	" O.	" "	
6-16-14.	" 140 "	" 1.7%	" "	
6-17-14.	" 290 "	" 4.0%	" "	
6-18-14.	" 270 "	" 4.4%	" "	
6-19-14.	" 410 "	" 3.6%	" "	Wt. 7.46 kilos.
6-20-14 to 6-30-14.	Continued diabetes. " " Wt. 6-29-14, 6.94 kilos.			
7-1-14.	Bright and lively, but emaciated. Abscess in neck. Sacrificed by bleeding under ether.			

Autopsy: Pancreas fragment appears normal. On microscopic examination islands of Langerhans are in a condition of advanced hydropic degeneration. Liver contains almost no glycogen. Adrenal medulla not remarkable.

OBSERVATION X. — Summary: Middle-aged bull terrier, female. Weight 10 kilograms. Removal of at least nine-tenths of pancreas. Transient glycosuria on meat diet. Transient glycosuria on meat and bread diet. Second operation and removal of about one-fifth of remaining fragment. Glycosuria at first moderate and then severe on meat and bread diet, persisting on meat diet. Third operation, unsuccessful attempt to ligate duct (no specimen removed). Permanent diabetes continued. Emaciation and weakness necessitated sacrifice of animal sixty-nine days after the beginning of the experiment.

3-25-14.	Urine free from sugar.	Operation.	Removal of all but a small portion of the pancreas, estimated at one-tenth or less about the main duct. Difficult, rather bloody operation.	No food this day.
3-26-14.	No urine.	Good condition.	Meat diet.	
3-27-14.	Urine 320 cc.	Sugar .77%	" "	Wt. 9.9 kilos.
3-28-14.	" 240 "	" O.	Meat.	Some bloody diarrhea.
3-29-14.	" 190 "	" O.	" "	
3-30-14.	" None passed.	Meat only.	Wt. 9.66 kilos.	
3-31-14.	" (48 hrs.) 240 cc.	Sugar 1.4%	Meat only.	
4-1-14.	" 240 cc.	Sugar 1.9%	Meat only.	Wt. 9.68 kilos.
4-2-14.	" 85 "	" O.		

4-3-14 to 4-7-14. No sugar in urine. Meat diet.

4-8-14. Urine 380 cc. Sugar 0. Meat and bread.

4-9-14. " 250 " " 1.66%. Meat and bread. Wt. 9.44 kilos.

4-10-14 to 4-14-14. No sugar in urine on meat and bread diet.

4-14-14. Second operation. Removal of cyst at upper end of remaining fragment of pancreas and excision of about one-quarter of remaining fragment. Specimens in fixation for examination. No food this day.

4-15-14. Urine 108 cc. Sugar 4.0%. Bread and meat diet. Good condition.

4-16-14. " 170 " " 1.0%. " " " "

4-17-14. " 460 " " .9%. " " " "

4-18-14. " 240 " " .6%. " " " "

4-19-14. " 140 " " .65%. " " " "

4-20-14. " lost. No data.

4-21-14. " 400 cc. Sugar .57%. " " " "

4-22-14. " lost. No data.

4-23-14. " 270 cc. Sugar .7%. " " " "

4-24-14. " 140 " " .7%. " " " "

4-25-14. " 500 " " 1.0%. " " " "

4-26-14. " 230 " " 1.25%. " " " "

4-27-14. " 450 " " 2.0%. " " " "

4-28-14. " 280 " " 2.0%. " " " " Wt. 8.8 kilos.

4-29-14. " 150 " " 1.25%. " " " "

4-30-14. " 430 " " 5.7%. " " " "

5-1-14. " 490 " " 4.3%. " " " "

5-2-14. " 340 " " 6.6%. Meat only.

5-3-14. " 300 " " 1.9%. " " "

5-4-14. " 500 " " 6.4%. " " Wt. 8.84 kilos.

5-5-14. " 250 " " 4.0%. " " "

5-6-14. " 350 " " 3.6%. " " "

5-7-14. " 175 " " 2.1%.

Third operation. Fragment of pancreas appears normal. No excision of specimen. Duct ligated and divided.

5-8-14. Urine 100 cc. Sugar 4.0%. Meat only.

5-9-14. " 160 " " 3.0%. " " "

5-10-14. " 240 " " 3.2%. " " "

5-11-14. " 220 " " 2.1%. " " Wt. 8.84 kilos.

5-12-14. " 180 " " .5%. " " "

5-13-14. " 420 " " 2.6%. " " "

Note: It appears at autopsy that the pancreatic juice in some way reestablished its course into the intestine. The increase in glycosuria from now on makes it appear that the juice found its way into the intestine at this time.

5-14-14. Urine 310 cc. Sugar 5.0%. Very little meat fed to-night. Wt. 8.04 kilos.

5-15-14. " 220 " " 2.1%. " " " "

5-16-14. " 260 " " .66%. " " " "

5-17-14. " 170 " " 0. " " " "

5-18-14. " 220 " " 1.9%. " " " "

5-19-14. Urine 90 cc. Sugar 4.0%. Much meat.

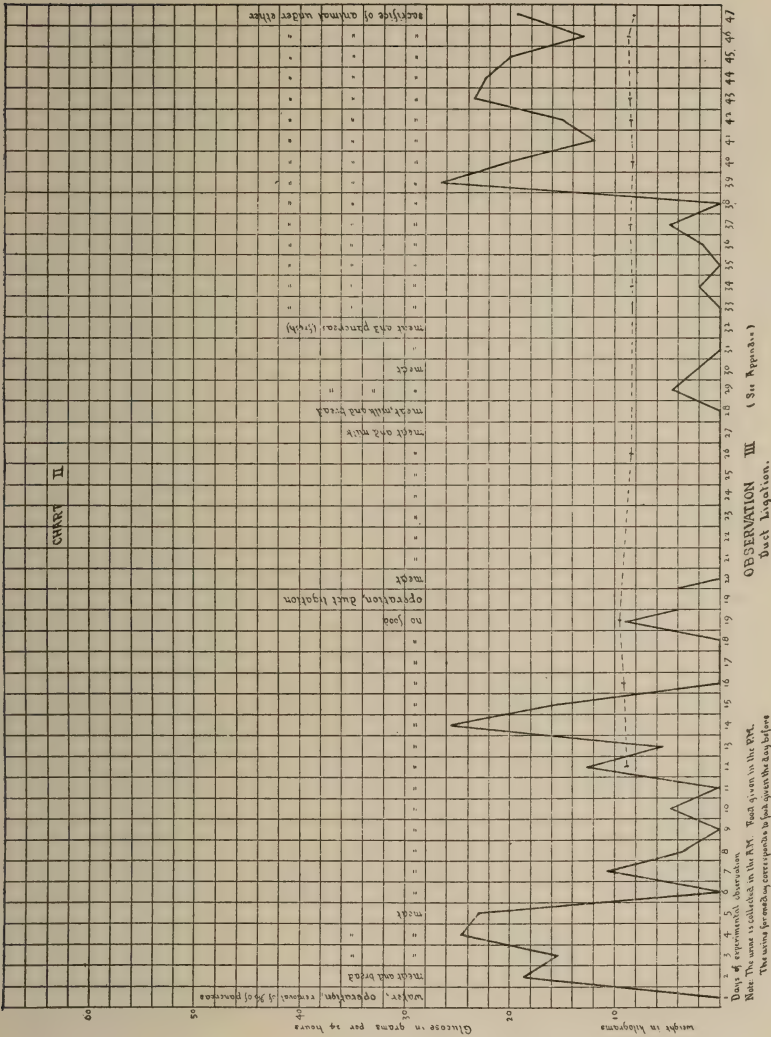
5-20-14. " 230 " " 4.0%.

5-21-14 to 6-2-14. Continued glycosuria on a meat diet, increasing in severity. Loss of weight to 7 kilos. Animal thin, but still active and ambitious. Sacrificed by bleeding under ether.

Autopsy: Duct remains occluded, but external secretion has evidently escaped into the intestine. Microscopic examination shows advanced hydropic degeneration of islands of Langerhans. Liver contains almost no glycogen. Adrenals, moderate exhaustion of adrenal medulla.

GROUP III. — Experiments demonstrating the physical effect upon the islands of Langerhans, in animals with a much-reduced amount of pancreatic tissue, of duct ligation, and the administration of fresh pancreas in the diet.

OBSERVATION III. (See Chart II.) — Summary: Adult mongrel, rather thin. Weight, 10 kilograms. Removal of an amount of pancreas estimated at nine-tenths of the gland. Bloodless, quick operation. Glycosuria upon meat diet, intermittent and varying in severity. Ligation of duct nineteen days after first operation. Following this, loss of weight but no glycosuria on meat diet, and mild glycosuria on mixed diet. Feeding fresh pancreas with meat caused return of glycosuria, which rapidly increased in severity. Gain of weight accompanied administration of pancreas at first, then loss of weight as glycosuria became more developed. Experimental diabetes demonstrated at autopsy. Advanced hydropic degeneration of islands of Langerhans.



11-18-13.	Operation. Removal of an amount of pancreas (29 grams) estimated at nine-tenths of the whole gland. No food this day.				
11-19-13.	No urine. Meat. Good condition.				
11-20-13.	Urine 620 cc.	Sugar 3.0%	Meat and bread.		
11-21-13.	" 530 "	" 2.9%	" "	" "	"
11-22-13.	" 720 "	" 3.3%	" "	" "	"
11-23-13.	" 820 "	" 2.8%	Meat only.		
11-24-13.	" 200 "	" 0.	" "	" "	"
11-25-13.	" 500 "	" 2.1%	" "	" "	Mild distemper.
11-26-13.	" 260 "	" 1.33%	" "	" "	" "
11-27-13.	" 360 "	" 0.	" "	" "	No "
11-28-13.	" 440 "	" 1.05%	" "	" "	"
11-30-13.	" 270 "	" 0.	" "	" "	"
12-1-13.	" 600 "	" 2.1%	" "	" "	"
12-2-13.	" 580 "	" 0.87%	" "	" "	"
12-3-13.	" 700 "	" 3.6%	" "	" "	"
12-4-13.	" 700 "	" 2.2%	" "	" "	"
12-5-13.	" 92 "	" 0.	" "	" "	Wt. 9.1 kilos.
12-6-13.	" 140 "	" 0.	" "	" "	"
12-7-13.	" 250 "	" 0.	" "	" "	"
12-8-13.	" 400 "	" 2.5%	Wt. 9.6 kilos.		

Second operation. Ligation of duct. Examination of specimens of pancreas shows general exhaustion of cells of islands of Langerhans, with slight hydropic degeneration in some islands (Figure 3). No food this day.

12-9-13.	Urine 900 cc.	Sugar 0.	Meat only.	Good condition.	
12-10-13.	" 400 "	" 0.	" "	" "	"
12-11-13.	" 460 "	" 0.	" "	" "	"
12-12-13.	" 330 "	" 0.	" "	" "	"
12-13-13.	lost.		" "	" "	"
12-14-13.	" 300 cc.	" 0.	" "	" "	"
12-15-13.	" 360 "	" 0.	" "	" "	Wt. 8.42 kilos.
12-16-13.	" 480 "	" 0.	Meat and milk.		
12-17-13.	" 340 "	" 0.	Meat, milk, and bread. Wt. 8 kilos.		
12-18-13.	" 220 "	" 2.0%	" "	" "	"
12-19-13.	not saved.		Meat only.	Diarrhea.	
12-20-13.	" 220 cc.	Sugar 0.	" "	" "	"
12-21-13.	" 280 "	" 0.	Meat and pancreas.		
12-22-13.	" 210 "	" 0.	" "	" "	"
12-23-13.	" 400 "	" .5%	" "	" "	Wt. 8.36 kilos.
12-24-13.	" 220 "	" 0.	" "	" "	Eats own feces.
12-25-13.	" 150 "	" 1.8%	" "	" "	"
12-26-13.	" 340 "	" 1.38%	" "	" "	Wt. 8.5 kilos.
12-27-13.	" 85 "	" 0.	" "	" "	"
12-28-13.	" 400 "	" 6.6%	" "	" "	"
12-29-13.	" 400 "	" 5.0%	" "	" "	Wt. 8.27 kilos.
12-30-13.	" 240 "	" 5.0%	" "	" "	"
12-31-13.	" 320 "	" 4.7%	" "	" "	"

1-1-14.	Urine 450 cc.	Sugar 5.2%.	Meat and pancreas.	Wt. 8.5 kilos.
1-2-14.	" 350 "	" 6.4%.	" " "	
1-3-14.	" 400 "	" 5.0%.	" " "	
1-4-14.	" 300 "	" 4.4%.	" " "	Wt. 8.4 kilos.
1-5-14.	" 340 "	" 5.7%.	" " "	" 8.2 "

Animal in good condition, but obviously diabetic and losing weight. Sacrificed by bleeding under ether.

Autopsy: Pancreas fragment very thick and firm. On section pancreas is white, opaque, but lobulations are distinct. (Usual appearance early after successful duct ligation.) Weight of fragment is 2.9 grams or less than one-tenth of original gland. Microscopic examination shows slight atrophy of acinous tissue with considerable duct proliferation. Islands of Langerhans in fairly advanced stage of hydropic degeneration. Liver, somewhat fatty; contains very little glycogen.

OBSERVATION V. (See Chart III. This observation is quoted in the text.) — Summary: Young, male Boston terrier. Weight 12.3 kilograms. Removal of an amount of pancreas estimated at more than nine-tenths of the gland. Rather long and difficult operation. Mild, temporary glycosuria on meat diet, followed by mild glycosuria on mixed diet. Duct ligation, and excision of specimen of pancreas for examination. Islands show evidence of over-activity with rare hydropic degenerated cells. After duct ligation, meat and pancreas cause no glycosuria. Meat and bread cause no glycosuria, but meat, bread, and pancreas bring on glycosuria which finally results in severe diabetes. Animal sacrificed forty-seven days after duct ligation. Autopsy shows advanced hydropic degeneration of the islands of Langerhans.

12-19-13.	Operation. Removal of an amount of pancreas estimated at nine-tenths. Weight 23.5 grams. No sugar in urine previous to operation. Vomited.			
12-20-13	Urine 850 cc. (partly vomitus).	Sugar .74%.	Meat only.	Good condition.
12-21-13.	Urine 280 cc.	Sugar O.	Meat only.	
12-22-13.	" 620 "	" O.	" "	Wt. 12.04 kilos.
12-23-13.	" 700 "	" .59%.	" "	
12-24-13.	" 500 "	" O.	" "	Wt. 11.23 kilos.
12-25-13.	" 550 "	" O.	" "	
12-26-13.	" 700 "	" O.	" "	Wt. 11.42 kilos.
12-27-13	" 680 "	" O.	" "	
12-28-13.	" 500 "	" O.	" "	
12-29-13.	" 480 "	" O.	Meat and bread.	Wt. 10.78 kilos.
12-30-13.	" 480 "	" .6%.	" " "	
12-31-13.	" 470 "	" O.	" " "	Wt. 11 kilos.

1-1-14.	Urine 360 cc.	Sugar 1.15%.	No food.	Wt. 11.42 kilos.
	Operation. Ligation of duct, and excision of specimens of pancreas for examination. Islands of Langerhans generally normal. Some show signs of over-activity and contain exhausted and hydropically degenerate cells (Figure 4).			
1-2-14.	Urine 420 cc.	Sugar 1.5%.	Meat and pancreas.	Wt. 10.8 kilos.
1-3-14.	" 380 "	" O.	" " "	Well digested movements.
1-4-14.	" 100 "	" O.	" " "	Wt. 10.8 kilos.
1-5-14.	" 300 "	" O.	Meat, bread, and pancreas.	" 10.58 "
1-6-14.	" 320 "	" 1.33%.	" " " "	" 11.2 "
1-7-14.	" 420 "	" 1.5%.	" " " "	
1-8-14.	" 400 "	" 2.58%.	" " " "	
1-9-14.	" 420 "	" 2.4%.	" " " "	Wt. 11.26 kilos.
1-10-14.	" 360 "	" 1.4%.	" " " "	
1-11-14.	" 210 "	" 2.1%.	" " " "	Wt. 11.3 kilos.
1-12-14.	" 450 "	" 1.9%.	" " " "	
1-13-14.	" 420 "	" 2.27%.	" " " "	
1-14-14.	" 280 "	" 3.5%.	Meat and bread.	Wt. 11.28 kilos.
1-15-14.	" 260 "	" .5%.	" " " "	
1-16-14.	" 320 "	" O.	" " " "	Wt. 11.36 kilos.
1-17-14.	" 350 "	" O.	Meat, bread, and stale pancreas.	Wt. 11.46 kilos.
1-18-14.	" 130 "	" O.	" " " " "	Diarrhea.
1-19-14.	" 300 "	" O.	Meat, bread, and fresh pancreas.	"
1-20-14.	" 320 "	" 1.25%.	Meat, bread, and pancreas.	Movements digested.
1-21-14.	" 400 "	" 3.6%.	" " " "	
1-22-14.	" 660 "	" 3.1%.	" " " "	
1-23-14.	" 340 "	" 3.3%.	" " " "	Wt. 11.62 kilos.
1-24-14.	" 480 "	" 2.6%.	" " " "	
1-25-14.	" 350 "	" 3.63%.	" " " "	Wt. 11.74 kilos.
1-26-14.	" 850 "	" 5.7%.	Meat and pancreas.	Wt. 11.6 kilos.
1-27-14.	" 135 "	" 2.0%.	" " " "	" 17.72 "
1-28-14.	" 130 "	" O.	Meat, bread, and pancreas.	Wt. 11.82 kilos.
1-29-14.	" 970 "	" 6.1%.	" " " "	
1-30-14.	" 770 "	" 8.8%.	" " " "	
1-31-14.	" 800 "	" 9.4%.	" " " "	
2-1-14.	" 470 "	" 8.0%.	" " " "	
2-2-14.	" 750 "	" 10.0%.	Meat and bread.	Wt. 11.6 kilos.
2-2-14.	" 400 "	" 10.0%.	" " "	
2-4-14.	" 160 "	" 8.0%.	Meat and pancreas.	
2-5-14.	" 105 "	" 3.8%.	" " "	Wt. 11.18 kilos.
2-6-14.	" 340 "	" 8.0%.	" " "	
2-7-14.	" 240 "	" 9.0%.	" " "	

2-8-14.	Urine 160 cc.	Sugar 11.4%.	Meat and bread.	
2-9-14.	" 315 "	" 11.4%.	Meat only.	Active and responsive.
2-10-14.	" 180 "	" 8.5%.	" "	Wt. 10.66 kilos.
2-11-14.	" 195 "	" 8.9%.	" "	
2-12-14.	" 220 "	" 10.0%.	" "	Wt. 10.34 kilos.
2-13-14.	" 180 "	" 10.0%.	" "	
2-14-14.	" 150 "	" 10.9%.	Meat and bread.	
2-15-14.	" 650 "	" 10.0%.	" " "	Wt. 10.22 kilos.
2-16-14.	" 500 "	" 10.0%.	Obviously weak, but responsive as ever.	
	Emaciated.	Weight 10.16 kilos.	Sacrificed by bleeding under ether.	

Autopsy: Pancreas fragment firm and scar-like. Weight 1.7 grams, or less than one-tenth of whole. Lobulations distinct. Duct occluded. Microscopic examination shows some atrophy of acinus tissue with proliferation of finer ducts. Islands of Langerhans show general advanced hydropic degeneration (Figure 7). Liver contains no glycogen. Adrenal. Considerable exhaustion of medulla.

GROUP IV. Experiments dealing with the effect of splanchnic stimulation upon glycosuria and upon the physical character of the islands of Langerhans.

A. Splanchnic stimulation with the pancreas intact: Three experiments were performed. The first two were tentative.

In the first, faradization was maintained for two hours only. A trace of sugar appeared in the urine within five minutes after the first splanchnic stimulation. The highest percentage of sugar was 4 per cent. The flow of urine rapidly diminished and sugar disappeared from the urine before the conclusion of the experiment.

In the second, faradization was maintained for three hours and fifteen minutes. A trace of sugar appeared in the urine after handling, and before division and stimulation of the splanchnics. The highest percentage of sugar was 2 per cent +. The flow of urine ceased after three hours, but the last specimen contained a trace of sugar.

The third experiment is described in the text.

B. Splanchnic stimulation with the pancreas reduced one-half. Two experiments were performed — both described in the text.

C. Splanchnic stimulation with the pancreas reduced four-fifths. The experiment is described in the text.

Control. Handling and division of the splanchnics (but no faradization) with the pancreas reduced four-fifths. This experiment is described in the text.

Histological technic. — A study of the pancreas by methods intended to demonstrate secreting granules demands the most minute care, more especially if comparisons are to be made between specimens of the pancreas removed at different stages of experimental disease. The following details are essential :

1. Post-mortem material must be removed instantly after the death of the animal. If removed more than an hour after death it is useless for study.

2. Specimens must be excised with a very sharp knife and with great gentleness.

3. Specimens must be not more than one to two millimeters in thickness, for the best granule fixatives have little penetrating power.

4. Sections for staining must be three micromillimeters or less in thickness, otherwise the deepness of the stains permits of no view of cell structure whatsoever.

The following fixatives have been found most useful in the study of secreting granules in the pancreas. Both will be found described in Bensley's monograph :

(a.) Acetic osmic bi-chromate :

Osmic acid 2 to 4%	4 cc.
Potassium bi-chromate 2.5%	15 "
Acetic acid (glacial)	2 drops
Mix freshly before using.	

Tissues are fixed in this solution from 8 to 24 hours, washed in water, and passed through alcohols to paraffin as in any other technic. The sections are stained by the anilin acid fuchsin-methyl green method.

(b.) Aqueous bi-chromate sublimate :

Corrosive sublimate	5.0 grams
Potassium bi-chromate	2.5 "
Distilled water	100. cc.

Tissues are placed in this fixative for from 8 to 24 hours, washed in water, and passed through alcohols to paraffin. The sections are stained in neutral gentian.

The following stains have been used in this study. The first has been most useful, and all drawings have been made

from sections stained by it. The second is less reliable but it is useful as a control to the first.

(a.) Anilin acid fuchsin — methyl green:

Anilin acid fuchsin is a 20 per cent solution of acid fuchsin in anilin water.

Anilin water.....	100 cc.
Acid fuchsin.....	20 grams.

Technic of staining. — Sections three microns or less in thickness are passed through xylol, absolute alcohol, dilute alcohols to water. They are placed for one minute in a one per cent solution of potassium permanganate and transferred at once for one minute to a five per cent solution of oxalic acid. They are then washed in water, and stained for six minutes in anilin acid fuchsin at 60° C.; washed thoroughly, placed for a few seconds in a one per cent aqueous solution of methyl green, and washed thoroughly in water. The success of the stain now depends upon whether the absolute alcohol in which they are dehydrated is free from water. The specimen is best washed with absolute alcohol from a drop bottle. If the alcohol is of good quality the red does not run in the specimen, but if the alcohol has any water in it, the red is extracted. When dehydrated the section is cleared in toluol and mounted in balsam.

A specimen of pancreas so treated has in general a blue to lilac color. The acinous cells are stained a smooth greenish blue; their zymogen granules are dark red; their slender mitochondrial rods bright red. The duct cells are generally uncolored except for their red mitochondrial rods, unless they contain granules such as are seen in the islands of Langerhans.

The islands of Langerhans have the appearance of a mass of granular material in which cell outlines are generally indistinguishable. The cells containing Bensley's "B" granules are slate-colored and always predominate in numbers over the cells containing Bensley's "A" granules, which are colored bright red, and so easily distinguished. Occasional uncolored cells, the undifferentiated cells of Bensley, are seen. All island cells contain mitochondrial rods, stained a bright red and much shorter and more irregular than the mitochondria of the acinous cells. The nuclei are rather irregularly stained in these preparations. They may be a dull green, or show a dark green outline about a clear space in which a bright red nucleolus is seen.

(b.) Neutral gentian: Neutral gentian is made by combining orange G and saure violet. A precipitate is formed in a neutral solution which can be collected and redissolved in absolute alcohol. A few drops of this saturated alcoholic solution added to twenty per cent alcohol gives the most satisfactory staining medium.

Sections freed from paraffin are passed through alcohols and placed for twenty-four or forty-eight hours in the dilute twenty per cent solution of

neutral gentian. They are then removed, blotted on filter paper, dehydrated in acetone, cleared in toluol and differentiated in a mixture of absolute alcohol one part, oil of cloves three parts. The heavy purple color of the section is extracted until it begins to appear brownish, and the decolorization is stopped by washing in toluol. The specimens are then ready to mount in balsam.

The acinous cells are stained a yellowish brown, while the zymogen in them stains a heavy purple. Duct and controacinous cells are unstained.

The islands appear a dirty blue, the granules of the "B" cells alone being stained. No mitochondria are shown. The nuclei are stained purple. Connective tissue, orange.

In general, the fixative for this stain penetrates so poorly that, unless the zymogen in the acinous cells is seen to be well preserved, no inferences should be drawn from the appearance of the island cells.

DESCRIPTION OF PLATES I.-II.

PLATE I., FIG. 1. — Normal resting island. Specimen removed at operation. (A) "A" cells. (B) "B" cells filling the greater part of the island. Note the absence of cell borders, the well-formed but not prominent nuclei, and the fine deeply-stained mitochondria standing out against a background of "B" granules. (Z) Masses of zymogen in the center of groups of acinous cells. The fine dark filaments in the free borders of the acinous cells are mitochondria. (RBC) Red blood corpuscle in a capillary. x 700 diameters.

FIG. 2. — Normal resting island. *Observation III.* (first operation): (A) "A" cell. (B) Group of "B" cells. Note indistinctness of cell outlines. Heavily-stained mitochondria are sharply outlined against a background of "B" granules. (Z) A mass of zymogen in a group of acinous cells. (RBC) Red blood corpuscles. x 600 diameters.

FIG. 3. — Moderate exhaustion and early "hydropic" degeneration of an island. *Observation III.*: Specimen removed nineteen days after the establishment of mild diabetes "gravis." Compare with normal resting island previously removed from the same animal (Figure 2). (A) "A" cell. (B) Crowded mass of "B" granules, among which are several cells undergoing "hydropic degeneration." (B¹) Group of exhausted "B" cells (note swelling of cells, clearness of outline, loss of granules, and some fusion of mitochondria). (B²) Early "hydropic" degeneration of "B" cells (note vacuolated appearance of the cells). x 600 diameters.

FIG. 4. — Functional activity, accompanied by exhaustion of several cells, and "hydropic" degeneration of one cell, of an island (a third, only, of the island is shown). *Observation V.*: Specimen removed twelve days after the establishment of a mild diabetes "levis." (A) "A" cells. (B) Active "B" cells (note prominence of nuclei and mitochondria, and visible cell borders). (B¹) Exhausted "B" cells (note loss of granules, fusion of mitochondria, and irregularity of the nucleus). (B²) "Hydropic" degeneration of a "B" cell. x 700 diameters.

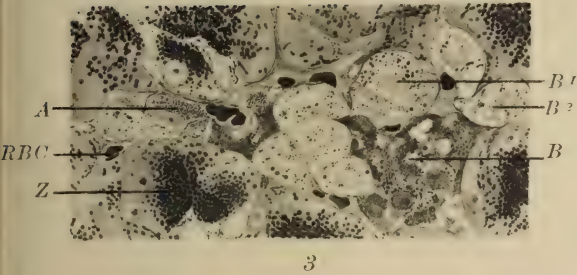
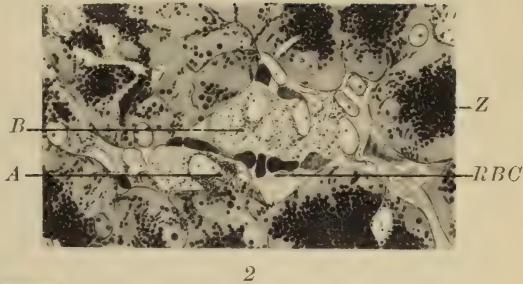
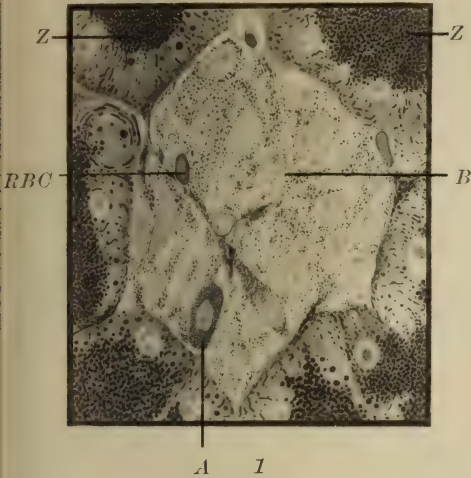
PLATE II., FIG. 5. — "Hydropic" degeneration of an island, moderately advanced. *Observation VIII.*: Autopsy (same as Figures 6 and 8). (A) "A" cells. (B¹) Exhausted "B" cell. (B²) "Hydropic" degeneration of "B" cells. (Z) acinous cells. (C) "Hydropic" degeneration of a duct cell (island cell?). x 700 diameters.

FIG. 6. — "Hydropic" degeneration of an island, moderately advanced. *Observation VIII.*: Autopsy (same as Figures 5 and 8). This animal showed a tendency toward recovery from a severe diabetes "gravis," but was sacrificed twenty-seven days after the disease became established (see Figure 8, drawn from the same specimen, for evidence of regeneration). (A) Normal "A" cells. (B¹) Exhausted "B" cells showing early stage of "hydropic" degeneration. (B²) "Hydropic" degeneration of "B" cells. (Z) Acinous cells loaded with zymogen. (C) Centro-acinous cell. x 700 diameters.

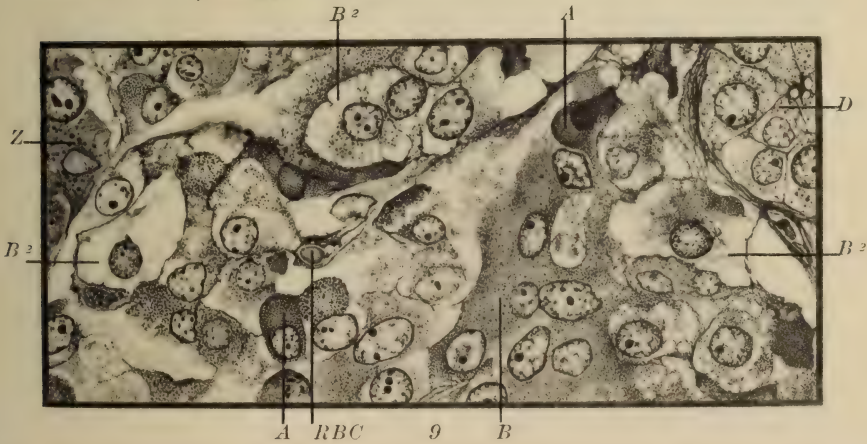
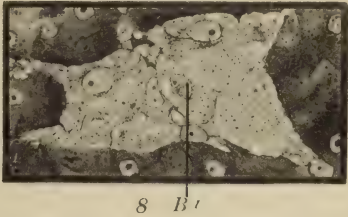
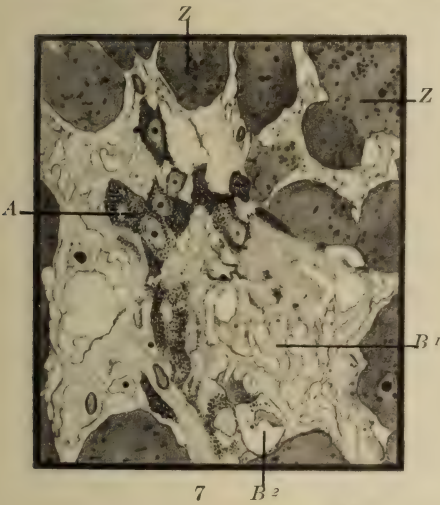
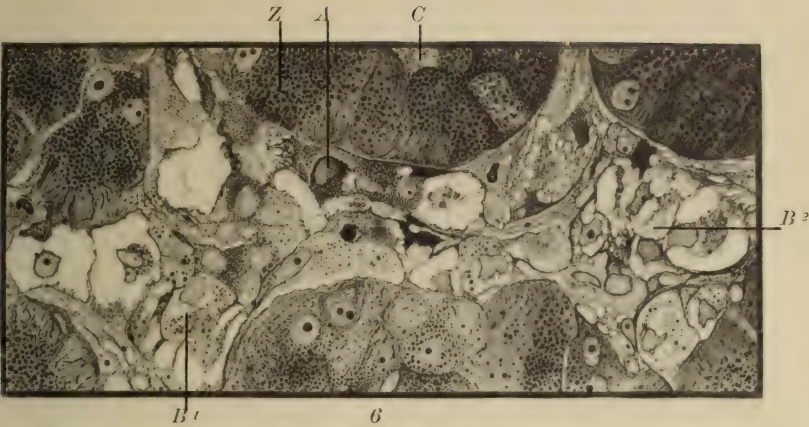
FIG. 7. — "Hydropic" degeneration, advanced. Island barely to be distinguished as such. *Observation V.*: Autopsy. Animal sacrificed forty-five days after duct ligation, and about two weeks after the establishment of permanent diabetes as a result of feeding fresh pancreas with carbohydrate food (see Figure 4 for earlier stage of the degenerative process in the same animal). (A) Well-preserved "A" cells, which never share in the degenerative process. (B²) "Hydropic" "B" cell, still recognizable. (B³) Mass of collapsed "B" cells, result of "hydropic" degeneration. (Z) Atrophied acinous cells. x 700 diameters.

FIG. 8. — Newly-formed (?) island. Compensatory regeneration. (B¹) Considerably exhausted "B" cells. Notice clearness of outline and well-preserved appearance of nuclei and mitochondria. No "A" cells are present in serial sections of this island. *Observation VIII.*: Compare with Figures 5 and 6, drawn from the same autopsy specimen. x 700 diameters.

FIG. 9. — Human diabetes of two years' duration: death from intercurrent disease. Early "hydropic" degeneration. The drawing represents only one corner of a very large island. Many islands appear normal, but in the majority, changes similar to those shown in this drawing are seen. (A) Normal "A" cells. (B) "B" cells, normal. (B²) "B" cells in various stages of "hydropic" degeneration. Compare these appearances with those shown in Figure 3, drawn from Observation III., an instance of mild diabetes "gravis" (mild glycosuria on a meat diet), and with Figure 4, drawn from Observation V., an instance of mild diabetes "levis" (mild glycosuria on a mixed diet). (Z) Acinous cells, containing a few zymogen granules and mitochondria. (RBC) Red blood corpuscle. (D) Duct cells. x 700 diameters.



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A STUDY OF THE SCHARLACH R REACTION AND OF ALLIED FORMS OF EPITHELIAL PROLIFERATION.*

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(*From Columbia University, George Crocker Special Research Fund,
F. C. Wood, M.D., Director.*)

PLAN OF PAPER.

I. THE WORK OF FISCHER AND OTHERS.

II. THE AUTHORS' EXPERIMENTS.

- (a.) *Detailed Study of the Early and Late Epithelial Reactions to Scharlach R Oil and Pine Tar Oil:* 1, Changes in epithelium at different stages; 2, Changes in connective tissue at different stages; 3, Changes in hair follicles.
- (b.) *Part Played by the Various Possible Factors:* 1, Initial injury; 2, Pressure and contact; 3, Irritant; 4, Solvent; 5, Tissue conditions.
- (c.) *Behavior of Tumor Cells.*
- (d.) *Effect of Scharlach R Oil, etc., on Glandular Epithelium:* 1, Breast; 2, Prostate and seminal vesicle; 3, Liver; 4, Lung.
- (e.) *General Discussion of Results:* 1, Initial injury; 2, Continued death and chronic irritation; 3, Chemical factors; 4, End result of epithelial activity; 5, Scharlach R reaction; 6, Contact and pressure; 7, Source of new epithelium; 8, Direction of growth; 9, Specificity and attraxine theory; 10, Atypical proliferation and tissue equilibrium; 11, Growth stimulus; 12, Lipoid solubility; 13, Metaplasia.
- (f.) *Conclusions.*

I. THE WORK OF FISCHER AND OTHERS.

The publication in 1906 of Fischer's³ experiments with Scharlach R, which announced the successful production of lesions simulating the histological picture of human epitheliomata, aroused much interest and furnished a stimulus for further experimental work along similar lines, chiefly as it suggested the possibility of the ultimate artificial production of tumors, and the study of their histogenesis. A survey of the literature covering the supplementary investigations which followed Fischer's original paper impresses one with

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the diversity of the results, and the variance of the views offered in explanation of the phenomenon. With the object of clearing up some phases of the problem, we have made a comprehensive study of the action of Scharlach R and certain other substances capable of eliciting epithelial proliferation. Our experiments, we believe, have proved that the atypical proliferation, caused by the injection of Scharlach R oil and similar substances, is a hyper-regenerative process, following an injury due to the application of a non-specific irritant under definite mechanical conditions. Further, we believe, we have also determined the importance of the several factors concerned in the reaction.

A brief summary of the literature will suffice here, since the general subject has been recently reviewed by Woglom,¹⁷ to whose work those interested are referred.

Fischer³ obtained his best results by injecting, under considerable pressure, a saturated solution of Scharlach R in olive oil, into the ears of rabbits just beneath the epidermis. The lesions produced were characterized by an increase in the thickness of the stratum Malpighii and stratum corneum, hyperplasia, and atypical proliferation of the hair follicles, disappearance of the sebaceous glands, and the later conversion of the epidermal and follicular epithelium into strands of epithelial cells, which invaded the underlying tissue, surrounding in their downward growth the oil droplets which they encountered. Fischer emphasized the necessity of injecting under considerable pressure. He stated that proliferation ceased when the oil was absorbed. Sudan III. and indophenol produced similar lesions. Fischer attributed the action of Scharlach R to a hypothetical epithelial "attractine."

Jores⁷ found that the germinal epithelium of the hair follicles took the principal part in the proliferative process when the injections were made under moderate pressure, and that the hypertrophy of the surface epithelium occurred only when the injections were made with considerable force. He also noted the difficulty of producing cancer-like lesions, and emphasized the importance of injecting into the proper tissue layer. Jores thought that the process began in the

cells of the hair follicles in the neighborhood of the sebaceous glands, and that the glands themselves played only a passive part, and soon disappeared amidst the proliferating tissue. The factors in the process, according to him, are the action of the dye on the upper portion of the hair follicles, and the proliferation of the epithelium which lies in contact with the oil globules. He explained the process as a regenerative one, exceeding physiological bounds.

Helmholtz⁵ showed that stratified squamous epithelium was not alone in its power to proliferate under the action of Scharlach R, but that the epithelial cells of certain of the organs, especially of the rectum, showed active proliferation after injection of the dye. He thought that the phenomenon might be due to interaction between some substance in the stain and the connective tissue, thus producing a soil appropriate for epithelial growth.

Levin⁸ stated that Scharlach R produced mainly epithelial proliferation in the rabbit, but that in the rat the dye acted only on connective tissue, no matter how or where employed.

Snow,¹² because he did not inject under pressure, was unable to duplicate Fischer's results.

It was shown by Stahr¹³ that a single injection of Sudan III., under high pressure produced atypical proliferation. He thought the results were due to a stimulus acting in a tissue of peculiar anatomical structure, and not to chemotaxis or the action of Fischer's attraxine.

A disturbance of normal nutrition due to obliteration of the blood vessels was the explanation given by Wyss.¹⁸

Meyer⁹ claimed to have produced the reaction with oil alone after the ligation of an artery or vein supplying the part, and thought that under such altered conditions the action of Scharlach R was accelerated. He believed the reaction due to chronic inflammation, associated with disturbances in the blood-supply.

Anemia and hyperemia were artificially induced in the ears of rabbits by Greischer and Schmincke,⁴ who found that the procedure did not alter the result of injection.

Stoeber¹⁰ confirmed the view held by Jores, that the chief

source of the new epithelium was the germinal cells of the hair follicles. With aminoazotoluol and Scharlach R he produced in the foot of an old man lesions resembling those produced in the rabbit ear, though not so extensive, because of the scant distribution of hair follicles.

Stoeber and Wacke¹¹ obtained epithelial proliferation with solutions of pyridin in olive oil, and with indol and skatol in rabbit fat. With the latter two substances they produced cancer-like lesions.

White¹⁶ obtained atypical proliferation with oleic acid alone, or saturated with carbon dioxide or methyl oxalate, in the ears of rabbits, the backs of mice, and the breasts of guinea-pigs. In the skin reaction he noted absence of an intrusion of epithelium and the non-participation of the hair follicles outside the inflammatory area: observations in opposition to Fischer's view of an attraxine. The lining of the abscess with epithelium is the result of the normal tendency of epithelium to grow over a free surface, and not to any specific property of the fatty acid. He thought that the injected material acted merely as a chronic irritant.

A number of different substances were used by Benthin² to induce atypical proliferation, all of which were inferior to Scharlach R and Sudan III. Paraffin and soot caused only the production of epithelial cysts. Oleic acid, glycerine, paratoluidin, and aminoazotoluol caused too much necrosis of tissue.

Benthin drew attention to the part played by the needle puncture, and laid stress upon the importance of contact of the chemical with epithelium. He referred the reaction to the several factors of inflammation, tissue tension, possibly circulatory disturbances, and last, but most important, the presence of certain chemicals.

On the assumption that the proliferation is due to some physical change in the lipid membrane of the cell, Wacke and Schmincke¹⁴ injected emulsions of tissue containing a fat-splitting ferment, but failed to elicit the reaction. In reviewing the various substances used to produce proliferation these authors found that with one exception, viz.: calcium carbonate, all of those which produced a positive result

were lipoid soluble ("lipoid löslich"), but they also discovered that some substances soluble or miscible in fats gave a negative result.

Herxheimer and Reincke,⁶ reviewing the subject from a theoretical standpoint based upon their own experiments and the experiments of others, concluded that cell proliferation is due to some change in the lipoid content of the cell.

Haga,^{4a} as the result of his investigations, concluded that the proliferation was due to prolonged chemical irritation which ceased upon absorption of the chemical. He decided also that the atypical reaction was in large part due to the relationship of the injected mass to the epithelium, and to the transplantation of epithelium by the needle.

II. THE AUTHORS' EXPERIMENTS.

(a.) *Detailed study of early and late epithelial reactions to Scharlach R Oil and Pine Tar Oil.*

Owing to the large number of animals used, detailed protocols have been omitted. Microscopic examinations were made of about 5,280 sections of tissue, derived from the following sources: Rabbits, 178 ears, 26 breasts; Rats, 31 lungs, 23 livers, 36 prostates, 36 seminal vesicles, 44 breasts, 89 skin, 11 tumors with Scharlach R sponge; Mice, 20 prostates, 20 seminal vesicles, 3 breasts, 122 skin, 18 tumors with Scharlach R sponge.

As an initial step in our experiments we have undertaken a detailed study of the early and late reactions following the injection of Scharlach R and pine tar, both of which have been considered as epithelial proliferants. When a saturated solution of Scharlach R in olive oil is injected into the skin of the rabbit's ear, just beneath the epidermis, and under considerable pressure, there is observed, about sixty to seventy hours later, considerable tissue destruction, inflammation and proliferation. The normal connective tissue at the place of greatest injury is replaced by a reticular substance composed of an irregular network of degenerated tissue and fibrin, holding in its meshes the globules of oil. This new tissue extends into the corium, and lies in contact with the epithelium of the hair follicles. Hemorrhage, occasionally

large deposits of fibrin, and polymorphonuclear leucocytic infiltration are also to be observed. Outside the area of degeneration there is dilatation of the smaller blood vessels, and an active proliferation of the mesothelial elements which inaugurates the final inclosure of the neighboring oil globules (Figure 1).

The epithelium shows varying degrees of injury, even undergoing necrosis at the point where it receives the brunt of the force of the injection. Here the epidermis shows some degeneration, and the lower parts of some of the hair follicles are completely destroyed. Bordering this area, the neighboring epidermis is slightly thickened, and there occurs hyperplasia of the upper part of the hair follicles, which is evidenced by the presence of mitotic figures in many of their cells. The hair canals show slight dilatation. The sebaceous glands apparently take only a passive part in the reaction. At the needle puncture, the hypertrophy is more marked and there is slight extension of the epithelial growth into the newly-formed tissue beneath.

After eight days a localized swelling is observed at the site of injection, surmounted by dry scaly skin showing a scant supply of hair. (The eight-day reaction is selected as the type of the active process, because the epithelial proliferation was found to be most active and extensive about eight days after the injection.) Microscopically, the reaction is characterized by extensive hyperplasia and downgrowth of the epithelium of the hair follicles. The transformed epithelium surrounds the larger collections of oil and, from the exterior of the epithelial shell, sends off along its course of growth numerous processes and strands of cells, which stretch into the depths or radiate outward in the loose, newly-formed connective tissue, towards similar processes from neighboring follicles, inclosing oil globules in their path. The epithelium may sometimes assume the form of a meshwork about the separate oil globules (Figure 2).

Associated with the extension of growth of the follicular epithelium is an increased formation of keratin and considerable desquamation of the cornified epithelial cells.

There is a strong tendency for adjacent follicles to unite, and, as will be seen in the later stages, with continued eccentric growth and concentric degeneration, a large cavity is produced, presenting several openings to the surface of the ear (Figures 3, 4). On cross section through certain areas, the transformed follicles are represented by central foci of keratinized cells surrounded by actively proliferating epithelium, producing the general picture of a squamous-cell epithelioma, as seen in the human skin. The epidermis also shows some increase in thickness and over-production of keratin, but this is not a marked feature. Many of the sebaceous glands have disappeared. The mesothelial cells engage actively in surrounding the foreign material, and the connective tissue becomes denser about the growing epithelium. The general picture gives the impression of a regenerative process, acting under irritation, in a tissue offering little resistance, and affords a demonstration of the part which epithelium may play under artificial conditions in clearing the tissues of noxious substances. The study of the later stages of the process strengthens this impression.

The early reaction (sixty to seventy hours) following forcible injection of a super-saturated solution of pine tar in olive oil differs from that of Scharlach R only in degree, there being more extensive degeneration of tissue and possibly greater proliferative activity on the part of the epithelial cells.

The later reaction (thirty to sixty days) shows conversion of the epithelium into numerous cysts of varying size, some single, others communicating with one another, or there may be a single immense cyst presenting numerous openings to the exterior. These cysts are lined by smooth, stratified squamous epithelium, resembling the epidermis, the cavities being filled with desquamated epithelium, tissue débris, and bits of pine tar (Figure 4). They are formed, as has been pointed out, by the growth of hair follicle epithelium around the foreign substance, and by the union of proliferating follicles, aided by the hollowing-out process due to keratin formation and desquamation. The finding of pine tar

within the epithelium and inside the cyst cavity as well, confirms our opinion as to the sequence of events. By employing pine tar oil containing *Lycopodium* spores, the fate of the foreign substance can be demonstrated even more distinctly.

About the cyst the connective tissue is dense and fibrous, showing the remaining scattered bits of pine tar in the process of being engulfed by the phagocytes. This late stage shows conclusively that the final outcome of the active process is the union of epithelium and the restoration of tissue equilibrium, an observation which strongly supports the view that the process is regenerative from the beginning. Metaplastic transformation of connective tissue into cartilage and of cartilage into bone, has been observed in ears injected several times with pine tar oil and excised seventy days after the initial injection. Particles of pine tar can be seen within the cartilage and about the cartilage and bone. The tissue changes were not followed beyond the seventieth day. Fischer, however, who followed the reaction for one year, records the complete disappearance of the epithelial cysts and their replacement by connective tissue.

(b.) *Part played by various possible factors.*

1. Initial injury. — It is to be noted that the many investigators in this field have failed to emphasize sufficiently the importance of the initial injury to epithelium and connective tissue produced by the prick of the needle, the force of the injection, and the action of the chemical, yet it is not improbable that by such injury the process is inaugurated.

Initial injury by needle: The insertion of the needle into the skin is the first step in the production of the reaction. Here, at the outset, two types of lesions are encountered, each a possible factor in the Scharlach R reaction. One lesion results from a puncture through the skin, while the other, located at a distance from the site of puncture, follows injury of the epithelium by the needle as it passes through the corium, in a direction parallel to the surface of the skin, involving also possibly the lower epidermal layers. The

participation of these two factors in the reaction depends, therefore, upon the proximity of the needle puncture to the site of injection, and the technic employed in the injection.

A simple needle puncture in a vascular area of the skin results in a slight hypertrophy of the follicular and epidermal epithelium adjacent to the puncture, and a slight downgrowth of delicate strands of epithelium into the blood-clot which is formed beneath. Injury to connective tissue likewise takes place, followed by active proliferation of the mesothelial elements and the final organization of the clot. As organization progresses, no mechanical obstacle to growth being encountered, the epidermis tends to follow its natural regenerative course and closes the gap in the tissue which was caused by the needle. The invading epithelium is lost amidst the connective tissue growth, and the normal condition of the tissues is reëstablished.

When the puncture is made into the skin overlying newly injected pine tar oil or Scharlach R oil, or when an injection is made in the immediate neighborhood of the puncture, so that there is contact between the surface epithelium and the injected substance, the epidermis contributes largely to the hypertrophy and proliferation. The much-thickened epidermis grows down along the track of the needle, forming a lining for the cavity in which the injected material is contained, surrounding separate oil globules, sending out strands of cells into the neighboring tissue, and forming a network about the oil droplets. The hair follicles of this region show active proliferation and participate in the encapsulation of the oil.

If, instead of a simple puncture being made, the needle be thrust for some distance into the corium, parallel to the surface of the skin, and just beneath the epidermis, there occurs death of cells of the hair follicles, and sometimes of the epidermis, followed by proliferation of follicular and epidermal cells. If the needle, introduced in the same manner, be twisted around in the tissues of the corium so as to produce more marked injury to epithelium and connective tissue, with the occurrence of small hemorrhages, the hypertrophy may be considerable, and the proliferation fairly extensive and

atypical. The reaction is confined chiefly to the cells of the epidermis (Figure 5). In contrast to the proliferation elicited by Scharlach R oil, little or no keratin formation is observed.

Injury by the needle and its resulting reaction is purely an incidental technical accompaniment of the Scharlach R reaction, and is in no wise essential to the production of the typical lesion. This is shown by the production of the reaction at a distance from the site of the puncture, and remote from the position in the tissues of the point of the needle at the time of injection.

Initial injury by forcible injection: That forcible injection is productive of injury is shown by the results of injecting either sterile physiological saline or plain olive oil under pressure. Sterile saline calls forth an inflammatory reaction of relatively moderate grade. There is some degeneration of the connective tissue and death of epithelium of the skin and hair follicles. Mitotic figures are fairly abundant in the epithelial cells, and cystic dilatation of the hair follicles occurs. Later stages (fifteen days) show thickening of the epidermal layer and some hypertrophy of the follicles. Active mitoses are still observed. The connective tissue is denser than in the earlier stages.

Olive oil injected under considerable pressure causes fairly marked injury to tissues, amounting even to actual loss of substance. There is some hypertrophy of the surface epithelium and hair follicles, the cells of which show mitotic figures, but there is no downgrowth into the underlying tissues. On account of the absence of a chemical irritant and the consequent relative inactivity of the epithelium, a much larger proportion of the oil is enclosed by mesothelial elements than is the case after the injection of Scharlach R at the same period, and degeneration, as well as fibrin formation, are less marked.

At the site of the needle puncture there is a greater degree of hypertrophy of skin and follicles, and limited atypical proliferation and downgrowth (Figure 6).

Initial injury by the chemical: A comparison of the effects produced by injections, under the same degree of

pressure, of plain oil and of Scharlach R oil, shows that the chemical is responsible for the greater part of the damage done to the tissues.

2. Pressure and contact. — Having shown the relative unimportance of forcible injection alone in the production of the initial injury, we shall consider forcible injection in another way. When Scharlach R oil is injected into the subcutaneous tissue of the ear under great pressure, there is little reaction of the epithelium, but when the dye is injected into the corium where it comes into contact with the epithelial cells of the hair follicles the typical lesion is produced. That the proliferation produced by forcible injection is dependent upon the contact of injected material with epithelium, rather than upon the effect of pressure and upon injury due to the force of injection, is shown by the results following two injections of small quantities of pine tar oil, made under minimal pressure at intervals of five days. The downgrowth and proliferation are not so extensive, owing to the limitation of the area of distribution of the pine tar solution, but in other respects they are similar to those following the injection of Scharlach R oil under pressure (Figure 7).

The importance of contact is well demonstrated by the results of the implantation beneath the skin of a small piece of sea sponge soaked in Scharlach R oil. Beginning at the point or points of contact between hair follicles and sponge, the thickened epithelium of the follicle splits into two parts (Figure 8). These grow irregularly in opposite directions around the sponge, sending processes or strands of cells into the overlying loose connective tissue, and uniting with the epithelium of neighboring follicles; or strands of cells may penetrate the tissue of the sponge, and surround the spicules of calcareous matter. The under surface of the proliferating epithelium adjacent to the sponge is covered throughout its extent by a thick layer of keratin, which serves as a protection against further destructive action of the dye, to which in great part it probably owes its origin.

Here there is seen the general tendency of epithelium to follow the path of least resistance, while the reaction also emphasizes the importance of giving the epithelium the right of way by temporarily checking connective tissue growth. The action of the chemical, aided by the expansion of the sponge, causes considerable death of connective tissue, and before reaction sets in and organization occurs, the epithelium has started on its course. The growth of epithelium does not extend into that part of the sponge which has become organized by the connective tissue, but follows a course in the unorganized portion of the sponge which offers less resistance. The election of this path of growth is probably due not only to the lessened resistance, but also to the fact that only in the unorganized portion of the sponge is the chemical free to act upon the epithelium.

Thus, of the three factors productive of the initial injury, the chemical apparently is alone essential. The injuries by needle and by pressure are only accessory, and need not enter at all into the reaction.

3. The chemical irritant. — In considering the chemical, we have sought to determine whether it is essential, or only an aid to the reaction, and whether bland oils or oily substances, aided by increased mechanical injury, would not cause lesions imitating those produced by Scharlach R.

In a previous paragraph we have shown that pure oil fails to elicit the reaction. However, by increasing the viscosity of the oil through the addition of beeswax, the epithelial reaction is slightly augmented. Vaseline, injected either cold or at a temperature slightly above its melting point, the skin over the injected material being pricked by a needle, produces a reaction similar in some respects to that of Scharlach R, though much less marked (Figure 9). The surface epithelium shows slight hypertrophy, which is most evident at the site of the needle puncture. The hair follicles are enlarged, cystic, and partly filled with epithelial squames. Outgrowths, in the form of processes or strands of epithelial cells, project from some of the follicles, or surround neighboring oil globules. The proliferation is generally confined to

the follicles concerned, and there is little tendency on the part of the epithelium of neighboring follicles to unite. The connective tissue of the cutis separating the follicles is rather dense and fibrous, and the oil droplets are scattered. The greater part of the injected material is found in the deeper tissue, and is partly surrounded by epithelium from the surface, which has passed down along the line of needle puncture. Strands of proliferating cells derived from this layer extend into the mass in an irregular manner. Contamination of the vaseline is suggested by the marked leucocytic infiltration. There is moderate formation of keratin.

The fact that we failed to duplicate the result by the injection of vaseline under the same condition, but omitting the injury to the skin by the needle, the factor of contamination also being present, indicates either an error in technic, or the blandness of the injected material. The active grade of proliferation and the formation of a moderate amount of keratin lead us to suspect the presence of some chemical irritant in the vaseline.

In order to insure contact a piece of sterile sea sponge, saturated with olive oil, was inserted subcutaneously, and the skin overlying the sponge was pricked with a needle. This resulted in a relatively slight downgrowth and atypical proliferation of the epithelium, which partially surrounded the spongy tissue.

Pressure, then, is necessary only to insure contact of the chemical with the epithelial cells, for without contact the chemical is without action on the epithelial tissue.

The chemical is necessary for extensive proliferation because, in consequence of its irritating action, death of epithelium is constantly occurring, followed by reparative cell proliferation. By the same power the chemical destroys connective tissue, and through its presence and action creates a disturbance of equilibrium between the two tissues, and a lessening of the resistance to epithelial growth.

Although the infliction of a single injury to epithelium in favorable tissue is conducive of considerable proliferation,

the reaction is much more limited than that caused by successive injuries, or, in other words, by injuries inflicted upon epithelium along its course of growth, such as occur after the injection of Scharlach R oil.

In the description of the Scharlach R oil reaction we have noted the concentric degeneration of epithelium and the eccentric proliferation. The dye itself, and not a deficiency of nourishment, is responsible for the greater part of the degeneration, as the following observations suggest: The extensive grade of keratinization about larger collections of the oil dye; the various stages of degeneration surrounding separate oil globules; the excessive keratinization of the lower layers of cells adjacent to the sponge saturated with the dye, and, finally, the degeneration of the basal cells of the free-growing ends. All these lead to the inference that the dye is responsible for the death of epithelium. Furthermore, the cornification observed in epithelial cells surrounding an abscess, such as is produced by aleuronat, is of relatively low degree, while that occurring in rapidly-growing epitheliomata seldom attains the degree and extent produced by Scharlach R oil; and regenerative epithelial processes, even if fairly extensive, are productive of but little keratin, in the absence of an irritant. Keratinization is most pronounced, in other words, after the injection of irritants known to cause cell death, such as pine tar and green soap (Figure 10). In the proliferation due to injury with a needle, cornification is relatively slight, or may be absent, but if the same degree of proliferative activity be excited by chemical injury, such as is observed after the injection of indigo, the extent of keratinization is considerable (compare Figures 5 and 11).

We may conclude that the formation of keratin is an indication of injury and ultimate cell death, dependent in greater part upon the chemical character and duration of action of the injurious substance, and to a lesser degree upon insufficient nourishment incident to excessive growth. The excessive cornification serves as a protection against further action of the chemical. In the keratin-lined cysts, after the

establishment of tissue equilibrium, the dye ceases to exert its action. Thus we believe that Scharlach R oil causes the death of those cells in contact with it, and that the more distant cells proliferate in excess to compensate for the loss; these in turn are acted upon and react in the same manner, and so on, until the foreign substance is surrounded. The process is rendered possible by the looseness or lessened resistance of the connective tissue, produced by the presence and action of the dye, and augmented by the widespread distribution of the dye. If the death of cells inaugurates and protracts the reaction we should expect, after the injection of very weak solutions of Scharlach R oil, either a retardation or a reduction of the reaction as compared with that called forth by strong solutions at corresponding intervals of time. It is very probable that weak solutions of the dye oil will cause death of cells after prolonged action, but owing to the necessity of a longer period of time to produce its destructive action, the proliferation should be less marked or delayed. Such is the case; weak solutions injected under the same degree of pressure and in the same quantity as the strong, produce a much less marked reaction (Figure 12). The degeneration observed in the cells, as well as the formation of keratin, shows that weak solutions are likewise capable of causing death of tissue after prolonged action.

An analysis of the results of the foregoing experiments explains the Scharlach R oil reaction. It has been shown that it begins after injury and cell death, and ends in cyst formation and tissue equilibrium; in other words, the innate power of injured epithelium to complete itself along the path of least resistance, is asserted. We have also seen that the irritant is constantly causing death of epithelium, and secondarily, therefore, the compensatory production of new epithelium. The facts that considerable proliferation of atypical character may be produced by purely mechanical means, and that the reaction can be inaugurated by bland substances, reinforced by mechanical injury, add weight to the explanation of the phenomenon as a process of regeneration.

As an additional argument in favor of the above explanation, it is appropriate to state here that when Scharlach R oil is injected into the more delicate skin of the mouse, there occurs considerable necrosis of epithelium and connective tissue, followed by an epithelial reaction which differs only quantitatively from that produced in the rabbit's ear.

Absorbability of the irritant: The next question regarding the irritant is that of its absorbability by the tissues, or the duration of its action upon epithelium. We have attempted to answer this question by a comparative study of the action of oily solutions of irritants (several of which have been used by other investigators) which vary in their rapidity of absorption. The tendency of irritants which are quickly absorbed to produce necrosis necessitated the use of weak solutions in oil, solutions less viscid than corresponding strengths of less absorbable irritant, and presenting greater difficulties in the maintenance of contact. Even with these weak solutions, however, necrosis was often encountered.

Pine tar, aminoazotoluol, skatol, iodine, turpentine, and eucalyptus oil were the substances used, with results varying more or less inversely with their absorbability.

The early (three days) reaction caused by aminoazotoluol (a substance chemically related to Scharlach R) in oil is essentially similar to that of Scharlach R oil. The typical lesion is not produced; the (eight to ten days) reaction resembles more closely that produced by vaseline. The later reaction following repeated (2) injections, which is less marked than that elicited by Scharlach R, consists of the formation of large single cysts as described under the late changes produced by pine tar. The failure to secure a typical reaction may have been due, however, to faulty technic, as other investigators have claimed success with this substance.

In our hands skatol in oil produced very little effect.

Iodine in oil causes tissue destruction, followed by considerable hypertrophy of the hair follicles and epidermis, adjacent to the injured area. Single injections fail to call forth the typical Scharlach R reaction, though they do

result in a certain degree of atypical proliferation. Two injections cause a generalized hypertrophy and atypical proliferation resembling that caused by vaseline, though more marked (Figure 13). The later stages of the reaction are characterized by epidermal cyst formation and fibrosis.

Turpentine in oil causes a general hypertrophy of the hair follicles and some growth into the subcutis, preceded by extensive death of both connective tissue and epithelium. Over the abscess there occurs hypertrophy of the epithelium, while at the site of the needle puncture the thickened epidermis turns inward to grow around the abscess, though showing but little tendency to invade it. In this area, and extending for some distance along the course of the invading epithelium, hypertrophy and active proliferation are observed. Here again is seen the tendency of epithelium to unite; follicles of the inverted skin join those above them; or proliferating processes from the lower-lying epithelium project upward toward similar processes of the upper follicles and surround neighboring oil globules. The connective tissue is dense about the abscess and there is active reaction about the oil droplets.

Eucalyptus oil, mixed with olive oil, causes much destruction of tissue. Outside of the necrotic zone hypertrophy of the epidermis and the hair follicles takes place, the latter showing moderate cystic dilatation. Scattered oil droplets observed in the subcutaneous tissues are enclosed by connective tissue cells. The later stages show a more or less irregular regenerative process. The dead connective tissue of the corium is replaced by dense fibrous tissue, and the area becomes covered with new epidermis devoid of hair follicles.

The results of these injections suggest that though irritants readily absorbed when mixed with oil do produce atypical proliferation resembling certain features of the Scharlach R reaction, they must not cause too great an injury in order to produce the widespread proliferation characteristic of the latter substance, and must, like pine tar, for example, remain in the tissues for a relatively long interval

of time (seven to eight days at least). The fact that repeated injections of iodine cause a much more marked reaction than a single one points to the need of a chronic irritant. That so many irritants are capable of causing atypical proliferation speaks strongly against the supposition that Scharlach R possesses a special affinity for epithelial cells; the results rather demonstrate the reactive power of epithelium in a tissue offering little resistance to growth.

Solubility of the irritant: Another factor bearing on the problem is the solubility of the irritant. With few exceptions, substances soluble or miscible in oil are insoluble or but slightly soluble in water, and, excluding the volatile irritants, the vast majority of these lipid-soluble substances are slowly absorbed by the tissues. A longer residence permits them to act upon the epithelium at all lines of contact along its course of growth. Substances soluble in water, on the other hand, are more or less rapidly absorbed, and, in consequence, their action upon the epithelium is of short duration and is confined to the region of initial contact. Substances insoluble in oil or oil solvents, and insoluble or but slightly soluble in water, resemble the lipid-soluble substances in their slow absorbability, and a consequent long sojourn in the tissues, and in the exertion of prolonged and widespread irritation.

A study of the reactions caused by the injection of substances belonging to each of the above classes may determine whether the production of cancer-like lesions of the skin is peculiar to lipid soluble irritants. The water-soluble irritants employed were injected alone, or in combination with a colloid substance in order to retard their absorption, the injury incident to injection being reinforced in some cases by needle pricks into the skin over the injected mass.

Classes of irritants: The following substances were used for the injections:

1. Lipoid soluble: Scharlach R (control).
2. Water soluble: Molasses, methylene blue (Grübler), yellowish eosin (Grübler), (skin punctured over the injected mass).

3. Insoluble in oil and insoluble or slightly soluble in water: Metallic mercury, aleuronat, charcoal (skin punctured), mercurous chloride, barium carbonate, calcium carbonate.
4. Allied to Classes 1 and 2: Glycerine, green soap, indigo.

Water soluble irritant: Extreme degeneration of the tissue with hyaline changes in the corium distinguish the lesions following the injection of molasses. There is some hypertrophy of the epidermis and hair follicles, and active cell division, the regenerating epithelium presenting but slight atypical features in its passage beneath the dead tissue. Dense fibrous tissue showing hyaline degeneration marks the later (fifteen days) reaction.

Methylene blue in egg albumen causes tissue death and limited hypertrophy of the epidermis and hair follicles, the hypertrophy being more pronounced in the region of the needle puncture. Absorption of the injected material apparently occurs early, none being found in the tissues after nine days. There is an active connective tissue reaction and little deviation from the normal in the regeneration of the surface epithelium.

Repeated (2) injections of eosin in thick egg albumen cause a more pronounced reaction. Owing to the fact that the injected material was in the subcutaneous tissues, little effect had been produced on the follicle cells. The reaction was chiefly confined to the epidermis, which passed down along the track of the needle to envelop partially the albumen, sending off strands of cells into the connective tissue to form delicate loops with the neighboring follicles, or to join the follicular epithelium above. Over the site of the injection the epidermis and hair follicles were moderately hypertrophied.

These results show that moderate epithelial proliferation may be brought about by the injection of substances apparently insoluble in oil, but soluble in water, and of relatively rapid absorbability, but that it falls far short of attaining the extent and degree of the Scharlach R oil reaction.

Substances insoluble in oil and oil solvents, and insoluble or but slightly soluble in water: Owing to the difficulty of obtaining contact between injected material and hair follicles, the results of the injection of metallic mercury were not especially suggestive. They may be summed up as follows: degeneration, slight hypertrophy of the epithelium, down-growth of surface epithelium about the mercury, and small cystic dilatations of the hair follicles. One of the injections resulted in the formation of a large abscess. Here the epithelial proliferation resembled that caused by turpentine in oil, though it was of more moderate degree.

Over the abscess formed by the injection of aleuronat, the skin and hair follicles show a certain degree of hypertrophy. The abscess itself is partially lined by epidermal epithelium, which passes inward along the path of the needle. The invading epithelium shows little abnormality and there are little or no actively proliferating hair follicles in this region, like those observed after the injection of turpentine oil. The epithelial cells bordering the abscess show a relatively slight grade of cornification. The surrounding tissues exhibit generalized inflammation, and the epithelium of the opposite surface of the ear is hypertrophied.

The reaction following the injection of charcoal suspended in water is so slight as to have no particular interest.

With the restriction that the epithelial reactions induced by the injection of calomel and barium carbonate, suspended in water, are most closely confined to the individual follicles and present a more or less localized peripheral growth, and, in the case of calomel, a greater degree of tissue death, the process resembles the Scharlach R oil reaction. The difficulty of obtaining contact between chemical and epithelium is great, but wherever contact was secured and injury was not too severe, atypical proliferation was encountered, certain areas even presenting a counterpart of the Scharlach R oil reaction in the radiating epithelial proliferation and the growth of epithelium around the foreign material.

The early reaction (seven to eight days), after the injection of calomel in water or in oil, presents areas of marked

atypical proliferation indistinguishable from the peripheral growth observed after Scharlach R oil (Figure 14).

The fifteen-day reaction following the injection of barium carbonate consists in the formation of a large, incomplete, compound cyst, presenting several openings to the surface, and derived from the epithelium of the hair follicles, as was noted after pine tar oil. (Barium carbonate was used at the suggestion of Dr. Shiro Tashiro.) Along the course of epithelial growth are observed areas of localized atypical proliferation (Figure 15). Calcium carbonate produces a reaction similar, but less extensive.

It is to be noted that the added injury to the skin was omitted in the introduction of these chemicals.

Judging from the results of these injections, it seems possible that a large number of inorganic irritants, insoluble in oil and water, could be collected, possessing the same proliferative action as Scharlach R, yet differing widely from one another in their chemical nature.

Substances allied to Classes 1 and 2: Glycerine causes sudden and extreme death of tissues, with a resulting epithelial reaction resembling that of eucalyptus oil.

Extensive and deep necrosis distinguishes the early changes following the injection of green soap; adjoining the area of dead tissue the epithelium is hypertrophied, passing beneath the necrotic mass and sending off irregular processes into the underlying tissues. The epithelium of the hair follicles participates in the reaction, presenting areas of atypical proliferating epithelium, resembling the active Scharlach R reaction.

The later reaction (fifteen days) shows irregular regeneration and cyst formation. There is an enormous overgrowth of cartilage, comprising in certain areas nearly half the thickness of the ear, which probably owes its origin to a normal regenerative process and to a metaplastic change of the connective tissues.

Indigo suspended in water or gum arabic produces a reaction qualitatively similar to the Scharlach oil reaction (Figure 16).

Reviewing the results of these injections, it may be affirmed that the power to induce atypical epithelial proliferation of the Scharlach R type is not confined to lipoid-soluble substances, but resides in inorganic substances insoluble in oil and having no affinity whatever for lipoids, that the degree of epithelial reaction varies inversely with the rapidity of absorption, and, finally, that a relatively mild irritant produces more extensive results than a powerful one.

4. The solvent. — Absorbability of the solvent: In regard to the question of the relative absorbability of the solvent, the same difficulties are encountered as were met in studying the irritant, that is, solvents of substances insoluble in water (bland oils excepted) are powerful irritants, and cause great destruction of tissue. Eucalyptus oil was chosen as one of the least irritating of the volatile solvents which are rapidly absorbed by the tissues.

The ten-day reaction, following the injection of Scharlach R in eucalyptus oil, is marked by extensive necrosis, and an invasion of the necrotic tissue by many leucocytes engaged in active phagocytosis. Outside of this area, and for some distance on each side, there is considerable hypertrophy of the epidermis and hair follicles, many of the latter being converted into cysts lined by keratin. Furthermore, there occurs union between some of the hair follicles and active eccentric proliferation. The picture resembles the Scharlach R oil reaction, although it is more limited, both in extent and in degree. Dense connective tissue is found beneath the proliferating epithelium. The dead tissue is not invaded by the growing epithelium, but is cast off by the growth from beneath, the hair follicles participating in the process.

Necessity of a solvent: It has been shown by other investigators that Scharlach R suspended in gum arabic produces a positive reaction when injected into the ears of rabbits, but only negative results have been reported after injection of the dye in water. In order to settle this question, the ears of rabbits were injected, under proper conditions, with a suspension of Scharlach R in water, and nine days later were excised

and examined in frozen sections. Ears of other rabbits were injected with Scharlach R gum arabic, and, finally, dry Scharlach R powder was rubbed into the ears of a third animal and buried in the tissues by pressure with a scalpel. This process is conducive of considerable inflammation and a certain amount of tissue death, due to the penetration of the epidermis by dye granules.

The reaction following the injection of Scharlach R water, as observed in frozen section, resembles qualitatively that caused by the injection of the dye in oily solution, but owing to the narrower limits of distribution, and the smaller quantity of foreign material, it is less extensive, and the proliferating processes are less numerous. Such sections are much easier to interpret than are those fixed and stained, in which the injected material is lost. Here, as was noted in the reaction following the introduction of a bit of sponge saturated with Scharlach R oil, the epithelium of the follicle splits in two to surround the collections of dye in contact with it, joining together again on the distal side of the foreign substance. Eccentric proliferation and concentric degeneration are observed. The participation of several follicles and the union of these follicles and their proliferating processes, combined with the hollowing-out process of degeneration and desquamation of epithelial cells, produce the typical picture, though on a smaller scale (Figure 18).

The injection of Scharlach R in gum arabic excites a reaction differing but little from the above.

The inunction of dry Scharlach R powder gives rise to lesions which imitate the dye-oil reaction, although they are superficial, and localized to the individual follicle. Collections of the dye just beneath the stratum corneum are surrounded by degenerated and keratinized cells, showing that even in solid form this material can attack the upper layers of cells when they are not protected by keratin. Other observers have claimed that the dye acts only on the cells of the stratum germinativum.

These experiments offer indisputable proof of the efficacy of Scharlach R to call forth a reaction, in the absence of a

solvent, differing only quantitatively from that elicited by Scharlach R oil. The difference in the two can be attributed solely to the physical properties of the oil, which permits a ready and widespread distribution in the tissues adjacent to the hair follicles.

5. Tissue conditions. — Anatomical structure: The character or type of the epithelial reaction is independent of the anatomical structure of the tissue concerned. The degree of the reaction is determined by the surface area of epithelium, exposed to the irritant throughout the duration of the reaction. Thus in a skin with a thick epidermal layer and a corium possessing large and numerous hair follicles the epithelial reaction is extensive. Because the skin of the rabbit's ear possesses these characteristics, and because of the facility it affords for securing and maintaining contact between chemical and epithelium, due to its structure, it is the site usually chosen for the injections. Yet the reaction, differing, if at all, only quantitatively from that produced in the rabbit's ear, can be elicited in the skin of the rabbit, rat, mouse, and probably in any animal at any site, if the proper technic be followed. We have been able, for example, to induce considerable epithelial proliferation in the loose tissues of the flank and side of the rat and mouse (Figure 19). The statement of Levin⁸ that epithelial proliferation cannot be produced in these animals is not supported by experiment.

Nutrition: Wyss¹⁸ attributed the proliferation caused by the injection of Scharlach R oil to an impairment of nutrition, following the obliteration of the blood vessels, and Meyer,⁹ likewise, thought the reaction was partly due to a disturbance of the blood supply. Greischer and Schmincke,⁴ on the contrary, found that neither anemia nor hyperemia influenced the reaction.

We have attacked this problem of nutrition from a somewhat different angle. If a smooth, absorbent cotton wick, saturated with Scharlach R oil, be passed through the tissues, so that the two ends of the wick project slightly beyond the surface of the skin, the epidermis at one end tends to grow

along the surface of the wick to join the proliferating epidermis from the opposite end. If, in place of the cotton wick, a wick composed of sea sponge be used, the epidermis not only grows along the surface, but penetrates the tissues of the sponge. By using such wicks as indicators, we have attempted to compare the degree of proliferation in epithelium deprived of a part of its blood-supply, with that of well-nourished epithelium.

Wicks saturated with Scharlach R oil were passed through the skin and subcutaneous tissues of rats and mice, and the areas containing the wicks were isolated from the surrounding skin by the denudation of circular zones about two millimeters in size; similar wicks were passed through the skin and tissues, constricted by means of purse-string sutures of thin wire tightly drawn together, and finally, wicks were passed through intact skin, as controls. The epithelial growth resulting from these different procedures presents little difference in extent and type, and there is no indication that poorly-nourished epithelium reacts to the influence of Scharlach R more actively than epithelium which is well nourished.

(c.) *The behavior of tumor cells.*

The behavior of tumor cells towards Scharlach R oil. — The behavior of tumor cells was studied by Werner,¹⁵ who found that a saturated solution of the dye in oil caused an acceleration of proliferative activity due, not to chemotactic influence, but to a true growth stimulus. Albrecht and Hecht,¹ however, treated tumor emulsion with powdered Scharlach R before inoculation and failed to induce excitation of growth.

The following experiment, based upon Fischer's attraxine hypothesis, was undertaken to determine what difference, if any, existed between the action of Scharlach R oil on epithelial and connective tissue tumors. If the hypothesis be valid, we might expect that the dye would exert a more profound influence upon carcinoma cells than upon those of sarcomata.

Mouse carcinoma and rat sarcoma were the tumors selected for the experiment. A sea-sponge wick, saturated with Scharlach R in oil, was passed through the skin and subcutaneous tissues adjacent to the tumor, or bits of sea sponge, soaked with the oil dye, were implanted subcutaneously by a trocar in close proximity to the tumor.

The animals were killed on the third, fifth, seventh, tenth, and fifteenth day after introduction of the sponge, and the skin, tumor, and sponge removed en bloc, fixed in Zenker's or Bouin's fluid, and cut in series of parallel sections about two millimeters in thickness, each section including a portion of skin, sponge, and tumor.

Macroscopical examination of the tumors at the stated intervals showed no indication of a localization of the growth on the side adjacent to the sponge, but rather more or less generalized growth throughout the circumference of the nodule. Microscopic examination showed some variability in the results with the individual tumors of the same type, but a general conformity in the lack of evidence of any chemotatic influence, and the indifference of both types of cells towards the foreign substance.

Where the sponge is situated within the capsule of the tumor and in close contact with the cancer cells, considerable necrosis ensues, due, possibly, not only to the action of the chemical, but to pressure atrophy, following expansion of the sponge tissue. Cells torn loose from the tumor during the process of inoculation, and planted elsewhere along the sponge borders, fared but poorly in their new surroundings, except in one instance, where the sponge appeared less compact; here growth was active.

When the wick or sponge lies adjacent to, but not in direct contact with, cancer cells, there is often no effect upon the tumor, but sometimes there is observed an inflammatory reaction of the connective tissue which involves the stroma of the tumor. Associated with inflammation is an increased vascularity of the tumor.

Invasion of the sponge tissue by cancer cells follows the

organization by connective tissue, or is a part of the general tumor growth.

Like carcinoma, sarcoma cells behave towards the "stimulus" in a passive manner. There is no tendency to change the general direction of growth towards the position occupied by the sponge, and only when partial organization of the foreign substance occurs do the sarcoma cells penetrate it.

(d.) *The effects of Scharlach R Oil, etc., on glandular epithelium.*

The discovery that pine tar oil and Scharlach R oil elicit a similar reaction of stratified squamous epithelium led us to extend the comparative study to other types of epithelium found in the internal organs. We selected the breast of the rabbit, and the prostate, seminal vesicles, liver, and lung of rats, organs showing a wide variability in the degree of cell differentiation and specialization. The mammary injections were made through the skin, or into the ducts by way of the nipple. The prostate, liver and seminal vesicles were injected directly through a laparotomy wound, while the lungs were injected through the chest wall or through the trachea. Concentrated oily solutions of Scharlach R and weaker solutions of pine tar were used, and the reactions noted after certain definite intervals of time.

1. Breast. — The result of injection into the mammary gland depends solely upon the technic, for here, as elsewhere, contact is necessary to call forth a reaction of the epithelium. To secure contact, the injection must be made through the nipple and care must be taken to avoid penetrating the wall of the duct; numerous injections made subcutaneously resulted only in a reaction of the connective tissue stroma of the gland. Both pine tar and Scharlach R cause an initial death of tissue, followed by proliferation of the epithelial cells lining the ducts. The dilated ducts may be lined by several layers of flattened cells or by an irregular membrane composed of heaps of cells, piled up in a disorderly fashion, one upon the other, which may completely fill the lumen. The innermost or more central cells show

varying stages of degeneration. There is also observed a meshwork formed by the union of strands of proliferating cells. In shape the cells are flat, rounded, or polygonal. A certain period of time after the injection of Scharlach R oil they may assume the shape and arrangement of those in the stratum Malpighii, showing the characteristic prickly cells and keratin; in fact, the metaplastic epithelium cannot be distinguished from excessively keratinized skin. Though the smaller ducts may participate, the reaction is observed best in the larger ones. The stage of transition is best seen in the pine-tar reaction, which does not reach the stage of complete transformation, though it is probable that a stronger solution of pine tar would produce the same condition. Sometimes the epithelial proliferation is so extensive as to surround oil globules outside the ducts.

The more highly specialized cells of the acini take a much less prominent part in the reaction. They are transformed into flattened cells which may extend in strands across the dilated acinus, but there is little active proliferation. Fischer, and also White, have described similar types of metaplastic change in the breast of the rabbit, following the injection of a solution of Scharlach R in ether.

From the standpoint of the clinician, these lesions are interesting in that they very closely simulate, histologically, certain types of chronic mastitis, as seen in the human mammary gland.

Other agents beside oily or ethereal solutions of irritants are capable of producing a reaction in mammary epithelium. Thus, this tissue will react also after the injection of metallic mercury; though the proliferation is much less pronounced, there occurs about the abscess following the injection, a well-defined transformation of duct epithelium into stratified squamous epithelium.

These results give weight to the view that cell death due to irritation is the factor of importance in the Scharlach R reaction, suggesting that the quality to which this dye owes its power of inducing cell proliferation is merely its power to destroy epithelium. It has not, contrary to the widely

prevalent opinion, any direct stimulative action. The proliferation of cells following the death of tissue suggests that the reaction here, as in the skin, is solely a process of regeneration.

2. Prostate and seminal vesicles.—The same difficulty of obtaining and maintaining contact of injected chemical and epithelium was encountered in the injections into the prostate and seminal vesicles, and the results were far from uniform. In some cases, the reaction was confined to the connective tissue stroma of the organs. Pine tar caused the same reaction in the vesicles as Scharlach R, but in the prostate the injection of pine tar resulted only in dilatation of the tubules and atrophy of the lining membrane.

The early changes in the prostate after the injection of Scharlach R oil consist of death and desquamation of epithelial cells, often hemorrhages, fibrin formation, and leucocytic infiltration. The tubules are filled with desquamated cells, débris, and blood elements. Regenerative growth takes place in the surviving cells of the tubules and the lost epithelium is replaced by a lining of cells one to several layers thick. Degeneration and vacuolization are observed in some of the cells. Later the thickened epithelium of some of the tubules is transformed into stratified squamous epithelium containing epithelial pearls. The connective tissue stroma surrounding the metaplastic tubules is denser than normal, presenting an inflammatory reaction about the oil globules.

The failure of pine tar to arouse the same reaction is due, probably, not to lack of power in the irritant, but rather to unavoidable variations in technic. The results of numerous injections of Scharlach R were very inconstant, some being absolutely negative.

In the seminal vesicles, pine tar oil and Scharlach R oil produce the same reaction. Again there is observed an irregular regenerative process following death of tissue. At the site of contact between injected material and epithelium there is observed a small irregular mass of cells, from which

emerge delicate strands of flattened cells which grow along the course of the injected material, forming loops with neighboring strands and enclosing oil droplets along their path. The growth is limited in extent and true metaplasia is not observed.

3. Liver. — Essentially the same results follow the injection of Scharlach R and pine tar oil into the liver. There is an initial degeneration of liver tissue and an active proliferation of the connective tissue stroma and the bile ducts. Proliferating bile ducts and islands, or strands of isolated liver cells, are found in the newly-formed tissue.

The liver cells show varying degrees of degeneration, and mitotic figures are occasionally seen. The later stages consist of the final encapsulation of oil globules and the replacement of dead tissue by connective tissue. The regenerative process is confined chiefly to the connective tissue and bile ducts and is probably no greater than that following other injuries of the same duration and degree. There is no indication that Scharlach R exerts a special action on liver cells.

4. Lung. — Injections of these two chemical substances into the lung call forth an active mesothelial reaction, and at the site of the perforation of the small bronchi an outgrowth of proliferating epithelial cells along the course of the injected material. There is considerable desquamation of the alveolar cells which apparently do not take an active part in the reaction. The later reaction resembles that observed in chronic inflammatory conditions, and consists of an increase in the connective tissue, and proliferation and metaplasia of the bronchial epithelium. Inflammatory metaplasia and proliferation of the bronchial epithelium are frequent findings in the rat lung and have, no doubt, complicated many of our results. At any rate, none of the injections caused a more marked reaction than is often observed in the lungs of chronic pneumonia, etc.

Injections into the lung through the trachea were likewise difficult of interpretation owing to this possible complication. None of the reactions were so typical as to be definitely

ascribed to the chemical. There is no doubt that the injection causes a chronic inflammatory condition of the bronchi and lungs with the resulting proliferation and metaplasia of the bronchial epithelium. The transformed epithelium is often arranged in strata and resembles the epidermal layer of the skin, but we have not observed keratin or intracellular bridges.

(e.) *Discussion of results.*

The results of the experiments described in this paper show that the epithelial reaction evoked by Scharlach R oil must be considered as a reparative process taking place under conditions of prolonged disturbance of equilibrium between epithelium and connective tissue and dependent for its inception and continuation on injury and cell death.

1. Initial injury. — Every successful injection of Scharlach R oil is followed by a certain amount of degeneration and death of epithelium and connective tissue, due to one or all of three possible causes, *i.e.*, the action of the dye, the force of the injection, and the injury by the needle. It has been shown that, although injuries by forcible injection and by the needle may enter into the reaction, they are not essential for the production of the typical lesion; for the dye alone, unaided by mechanical injury, is capable of eliciting extensive atypical proliferation. Since, after the introduction of Scharlach R, cell death always precedes cell proliferation, and since the last two of the three possible causes of cell death have been eliminated, the obvious conclusion is that the dye is responsible for the initial death of tissue.

2. Continued death and chronic irritation. — There is no cessation of the activity of the dye after the initial death of tissue; on the contrary, there is a prolongation of its toxic action, which results in a continued overproduction of keratin and a compensatory formation of new epithelium. The overproduction of keratin is an indication of cell death, and it is due in great part to the action of Scharlach R, rather than to an impoverishment of nutrition. This has been

previously explained by the excessive cornification occurring after the injection of substances known to cause cell death, such as indigo, pine tar, and green soap. The keratinization of the epithelial cells about the dye granules embedded in the epidermis cannot be explained by assuming a lack of nutrition, since there is no deficiency. Finally, it is to be emphasized again that, in the absence of an irritant, regenerative processes are productive of but little keratin, even when they are fairly extensive. It may therefore be declared that Scharlach R is not only the cause of the initial injury, but also that it is responsible for the greater part of the tissue death which is occurring constantly during the reaction. That a chronic irritant capable of causing constant death of epithelium is requisite for the production of extensive proliferation has been demonstrated in the relative inefficiency of single injections of volatile substances, or of substances readily absorbed; whereas when such irritants are injected repeatedly the reaction produced resembles more closely the Scharlach R reaction. Hence, Scharlach R acts in the tissues like a chronic irritant, producing prolonged and widespread injury and death of tissue. The dye cannot act through excessively keratinized cells, which thus protect the epithelium against its further action.

3. Chemical factors. — It has been shown that, although certain bland substances aided by mechanical injury can inaugurate the reaction, and that atypical proliferation can be produced by simple mechanical injury, the typical reaction depends upon the presence of a chemical possessing the properties of a relatively mild and slowly absorbable irritant. Such irritants, as green soap, for example, which cause an initial extensive destruction of tissue, are undoubtedly rendered less toxic by the rapid absorption of the highly irritating part of the chemical, and possibly by some neutralizing action of the tissues, which thus leaves the milder irritant free to act upon the tissues.

4. End result of epithelial activity. — Fischer has stated that the Scharlach R reaction ceases after the absorption of

the injected material, but we have shown that the cessation of proliferation antedates absorption, and is an indication of the completion of the new task imposed upon the tissues. This task implies the encapsulation of the foreign substance and a return to tissue equilibrium. The later changes in the epithelium are simply regressive in character.

5. The Scharlach R reaction. — Only one conclusion can be drawn as to the nature of the reaction: the process elicited by Scharlach R is a reparative (hyper-regenerative) one. A process which begins only after injury and cell death, which depends upon cell death for its continuation, and which ends in tissue equilibrium, must be considered as regenerative. In addition, when one considers that the inauguration of the process can be brought about by bland substances, aided by mechanical injury, no other interpretation of the reaction appears even plausible.

6. Contact and pressure. — Contact between chemical and epithelium is requisite, for without contact no death of epithelium occurs. The degree of proliferation depends upon the area of contact maintained throughout the duration of the reaction.

Pressure or forcible injection, the factor so much insisted upon by Fischer, is in itself unessential for the reaction, and serves mainly to secure a wider area of contact.

7. Source of the new epithelium. — Jores and others contend that the new epithelium is derived principally from the germinal cells of the hair follicles, and that the participation of the epidermal cells in the reaction depends upon the force of the injection. In reality, the source of the new epithelium depends upon what parts are involved in the degeneration. If the lower parts of the hair follicles be destroyed, the proliferation begins in the healthy cells adjacent to the area of degeneration; if the entire follicle be destroyed the epidermal cells about the mouth of the follicle furnish the new epithelium; and if both follicle and epidermis be destroyed, the neighboring follicle cells and the

sound epidermal cells assume the task of regeneration. This is observed best in the less resistant skin of the mouse and rat, or in the ears of young rabbits.

8. Direction of growth. — There is an inherent tendency for severed epithelium to join together along the path of least resistance, and, whenever resistance is encountered, to deviate from its natural course towards a path which opposes no barrier to growth. There is a lesser proclivity for epithelium to surround or encapsulate foreign materials with which it comes in contact, though such a tendency is enhanced if the foreign substance be an active irritant.

Following the injection of Scharlach R powder in water, there occurs death of the epithelial cells (hair follicle cells, for example) and a replacement of the normal connective tissue adjacent thereto by the foreign irritant. The dead cells and the dye, which is packed closely against them, supply the barrier against the normal course of regeneration and the epithelium, influenced by the tendency to surround the foreign body and hindered from following the natural course by the mechanical obstruction, attempts to perform that task. Under continued irritation and death of tissue, the task is accomplished. With the constant death of epithelium there is an active overproduction of new epithelium, no doubt rendered possible by the looseness of the connective tissue. Thus the growth of epithelium about the dye is similar to the growth about the sponge saturated with the dye, and is, in part, comparable to the growth of epithelium along the surface of the wicks passed through the tissue, and projecting beyond the surface of the skin.

9. Specificity and attraxine theory. — It is scarcely necessary to touch upon the attraxine theory promulgated by Fischer, or the question of the specificity of Scharlach R, since they have been answered by the results of our experiments. The so-called specific property of Scharlach R, which is possessed, in fact, by other irritants to at least an equal degree, is due to nothing more than the relative mildness of the irritant, and to its slow absorbability, and if an

attractine be ascribed to this dye, a similar possession cannot be denied to countless other materials, including even substances ordinarily considered free from irritating qualities.

The results of our experiments conflict in no wise with the opinions of surgeons and clinicians concerning the therapeutic value of Scharlach R in hastening the epidermization of healthy granulating wounds; they merely show the mode of action of the dye, and furnish an explanation of the reason for that action. The ease with which extensive epidermal downgrowth is incited, however, warrants a word of caution as regards the practical application of the drug. Although in none of our experiments did a tumor result, it is not impossible that prolonged application, and especially injection about the margin of a chronic ulcer, might finally induce a malignant change in response to the chronic irritation.

10. Atypical proliferation and tissue equilibrium. — The description of the reactions produced by the various methods shows the almost constant occurrence of invasive epithelial proliferation, and brings up the question of the conditions which determine whether the proliferation is to be normal or atypical. We have observed atypical proliferation after injury to the skin and underlying tissues by the needle, and an accentuation of the process occurs, following an injury to the skin overlying a bland foreign substance. A still greater degree of atypical proliferation is produced by the injection of irritants in oil and by certain irritants alone. Attention has frequently been called in this paper to atypical proliferation of the hair follicles of the inverted skin at the site of needle puncture over injected irritants, and a somewhat similar reaction was noted in the same locality over an abscess produced by the injection of metallic mercury. In all these areas the connective tissue adjoining the proliferating epithelium showed some evidence of rarefaction. The occurrence of a very similar epithelial proliferation has frequently been noted at the edges of chronic ulcers, and the epithelial invasion of the corium in X-ray keratoses resembles very closely the experimental results in the rabbit's

ear. These, and other examples which need not be given in detail here, suggest a common factor—disturbed tissue equilibrium. If the connective tissue be inflamed, and thus rendered more spongy, a slight irritation of the epithelium by any chemical substance or physical influence may be followed by atypical invasion of the altered connective tissue by the epithelial cells.

We may, therefore, consider that invasive epithelial proliferation is the result of an injury and death of epithelium in tissues out of mechanical equilibrium, and that the degree and duration of the proliferation are dependent upon the extent and persistence of action of the injurious agent and upon the amount, duration, and, possibly, the variety of the connective tissue disturbance.

Insurmountable difficulties would be encountered were an attempt made to prove conclusively that the difference in tissue tension, or, in other words, the lessening of the normal mechanical resistance to invasive epithelial growth offered by the connective tissue, is due to the prolonged action of the dye upon the connective tissue, and not simply to the presence in the tissues of the foreign substance. Owing to the fact that the chemical acts apparently just as freely upon the connective tissue as upon the epithelium, and that degeneration occurs concurrently in the two tissues, we have not been able to devise an experimental method of settling this question.

II. Growth stimulus. — We shall enter into the old question whether growth implies a direct growth stimulus only as it concerns the action of Scharlach R. Our results are in harmony with the view of Weigert, which regards reparative processes, not as the result of a direct stimulus, but rather as a consequence of the removal of influences which prevent growth. The Scharlach R reaction is a reparative process, rendered possible by removal of the resistance to invasive epithelial growth normally offered by connective tissue, and due to the presence of the dye. What physical or chemical changes occur in the cell are still unknown, but if Scharlach

R be considered a true stimulus to growth, then all irritants must be placed in the same category.

12. Lipoid solubility. — Certain German investigators explain the action of Scharlach R as the result of some change produced by the dye in the lipoid content of the epithelial cell, and include under the term "lipoid löslich" a large and varied assortment of substances possessing the power to provoke epithelial proliferation. These authors note also that a few "lipoid löslich" substances do not possess this faculty, and that the fat-splitting enzymes isolated from lymphoid tissue are likewise ineffective.

The possibility that the lipoid membrane of the epithelial cell may undergo some physical or chemical change previous to cell division cannot be denied, but the statement that irritants owe their action to their power to dissolve lipoids is purely theoretical, and lacking in experimental proof. Yet an hypothesis which attempts to explain the proliferative action of irritants should be of universal application, and free from exceptions. We have pointed out these exceptions, and have shown that several, and, possibly, many irritants which are not lipoid soluble possess powers very similar to those of Scharlach R and other lipoid soluble substances. We have also pointed out that materials which possess this power in the highest degree exert a relatively mild effect and are but slowly absorbed, characteristics to which it seems entirely reasonable to refer their activity, since this activity is in all probability due to these qualities, rather than to lipoid solubility. Indeed, the effectiveness of all classes of irritants, both lipoid soluble and insoluble, may be explained in this way.

An apparent exception to our argument may be found in the reaction of Scharlach R in eucalyptus oil and ether, as these solvents are volatile substances which are rapidly absorbed by the tissues, leaving the greater part of the dye in the tissues in an insoluble form. This may be explained by the fact that Scharlach R powder possesses powers identical with Scharlach R oil, and that when the volatile part is absorbed, the solid particles of the dye left in the

tissues may continue to exert their action upon the undestroyed and regenerating epithelium.

13. Metaplasia. — We have observed the transformation of the epithelium of the hair follicles into cornified epithelium under the influence of the dye, and have noted the same change in the prostate gland and in the ducts of the breast. Incidentally, the transformation of connective tissue into cartilage, and of cartilage into bone, has been observed in some of our experiments after repeated injections of pine tar oil into the ears of rabbits. Metaplasia is frequently seen in chronic inflammatory processes and in mucous membranes exposed to injury. Wherever encountered, aside from physiological processes, it is an indication of chronic inflammation or irritation, and is probably often a protective process, the reversion of one type of cell to another which affords greater resistance to the action of an injurious substance.

(f.) *Conclusions.*

From the experimental data recorded we feel justified in drawing the following conclusions:

1. The Scharlach R reaction is a reparative process, acting under conditions of prolonged disturbance of mechanical equilibrium between epithelium and connective tissue, and dependent for its inception and continuation upon irritation and cell death.

2. The contact with epithelial cells of a chemical irritant of slow absorbability and relative mild toxicity is indispensable for the production of the reaction.

3. The chemical owes its power of inducing epithelial proliferation, not to its solubility in lipoids, but to its ability to cause continuously a certain amount of tissue death.

4. Cessation of epithelial proliferation occurs before the absorption of the foreign substance, and indicates the return of equilibrium after the performance of the new task imposed upon the tissues.

5. The character and extent of the reaction are independent of the amount of blood circulating in the tissues. The

degree of reaction depends upon the surface area of epithelium exposed to the irritant.

6. Scharlach R exerts no specific stimulating action upon epithelial cells.

7. The attraxine hypothesis of Fischer is untenable.

8. Metaplasia is a frequent occurrence in the tissues of the ear, breast, prostate, and lung, after the injection of a chronic irritant of the type used in our experiments.

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DESCRIPTION OF PLATES III.-IX.

PLATE III., FIG. 1. — Reaction in rabbit ear, 67 hours after injection of pine tar oil. Note: A, marked degeneration of both epidermis and hair follicles.

FIG. 2. — Reaction in rabbit ear, 8 days after injection of Scharlach R oil. Note: A, union of proliferating follicles and their processes; B, commencing cyst formation.

PLATE IV., FIG. 3. — Reaction in rabbit ear, 10 days after injection of Scharlach R oil, showing cyst formation. The opening to the surface is not shown. Note the union of hair follicle epithelium with that of the upper surface of the cyst wall.

FIG. 4. — Same as Fig. 2, with more marked cyst formation at A.

PLATE V., FIG. 5. — Epithelial proliferation following injury to epidermis by needle (reaction after 5 days).

FIG. 6. — Reaction in rabbit ear, 15 days after injection of olive oil under pressure. Site of needle puncture.

FIG. 7. — Reaction in rabbit ear, 15 days after first of two injections of pine tar oil under minimal pressure.

PLATE VI., FIG. 8. — Reaction in rabbit ear, 10 days after implantation of sponge soaked in Scharlach R oil. A, sponge infiltrated with leucocytes. B, proliferating epithelium of hair follicles. Note keratinization and degeneration at under surface of proliferating epithelium.

FIG. 9. — Reaction in rabbit ear, 10 days after injection of vaseline with added mechanical injury.

FIG. 10. — Marked keratinization in rabbit ear, 12 days after injection of green soap. A, keratinized zone which surrounds injected material; this last is not shown. B, surface epithelium.

PLATE VII., FIG. 11. — Reaction in rabbit ear, 8 days after injection of indigo. Note: A and B, cornification.

FIG. 12. — Reaction in rabbit ear, 8 days after injection of weak solution of Scharlach R oil.

FIG. 13. — Reaction in rabbit ear, 15 days after first of two injections of iodine in oil.

PLATE VIII., FIG. 14. — Reaction in rabbit ear, 10 days after injection of calomel in oil. Epithelial proliferation.

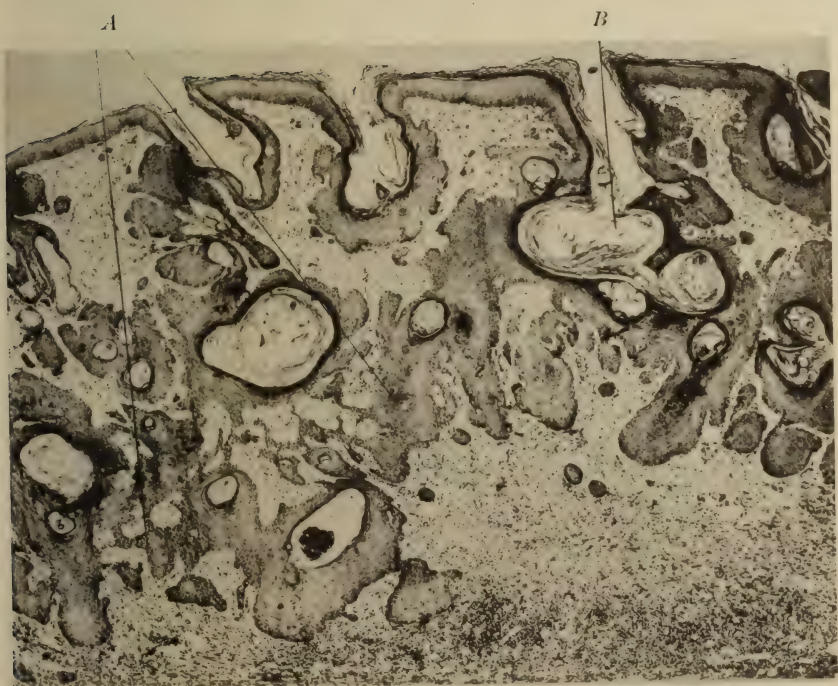
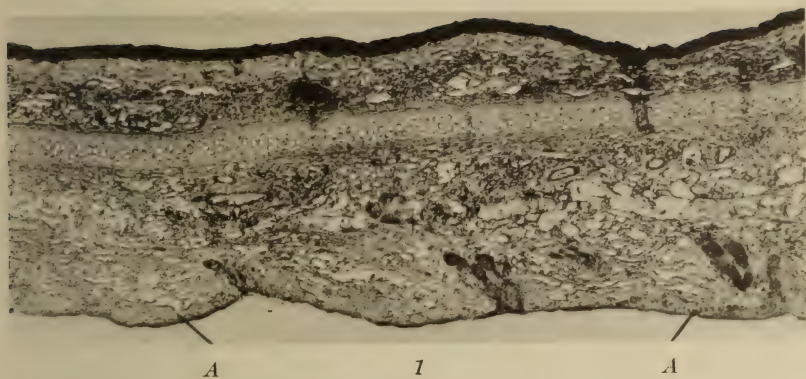
FIG. 15. — Reaction in rabbit ear, 15 days after injection of barium carbonate in water, showing one cyst, A, with two openings, and part of another cyst, B.

FIG. 16. — Reaction in rabbit ear, 9 days after injection of indigo gum arabic.

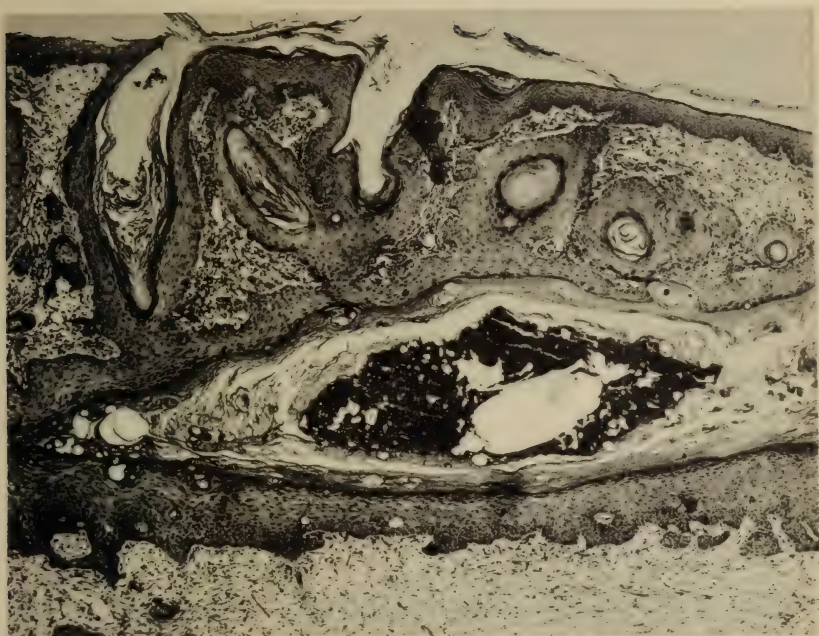
PLATE IX., FIG. 17. — Reaction in rabbit ear, 10 days after injection of Scharlach R in eucalyptus oil.

FIG. 18. — Reaction in rabbit ear, 9 days after injection of Scharlach R in water (paraffin section).

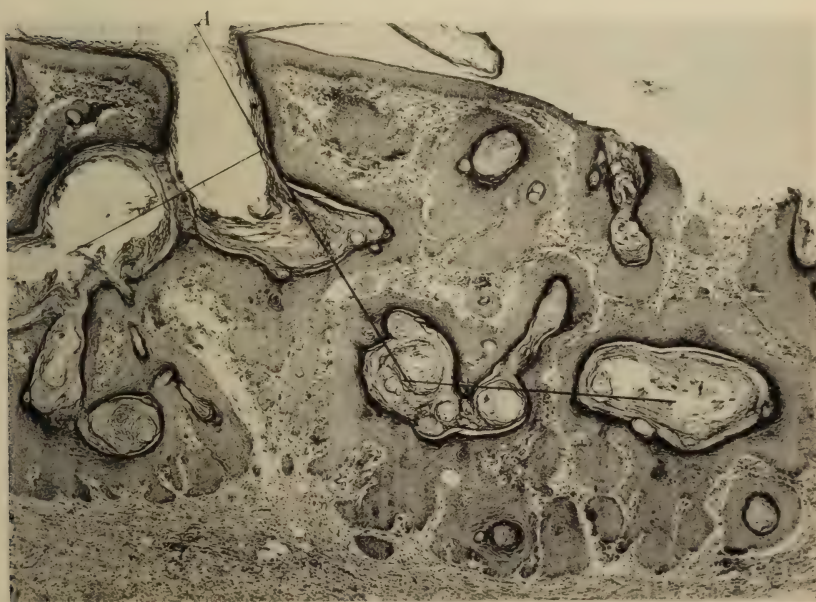
FIG. 19. — Reaction in mouse skin, 12 days after injection of Scharlach R oil. Note: A, hypertrophy of epidermis; B, cyst formation.



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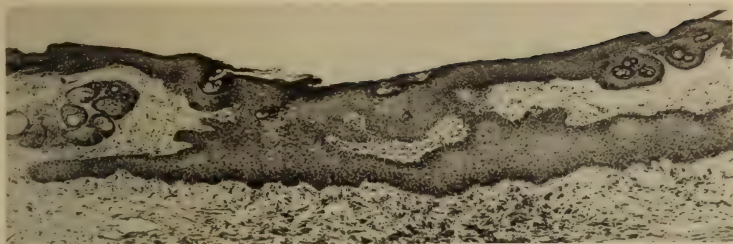


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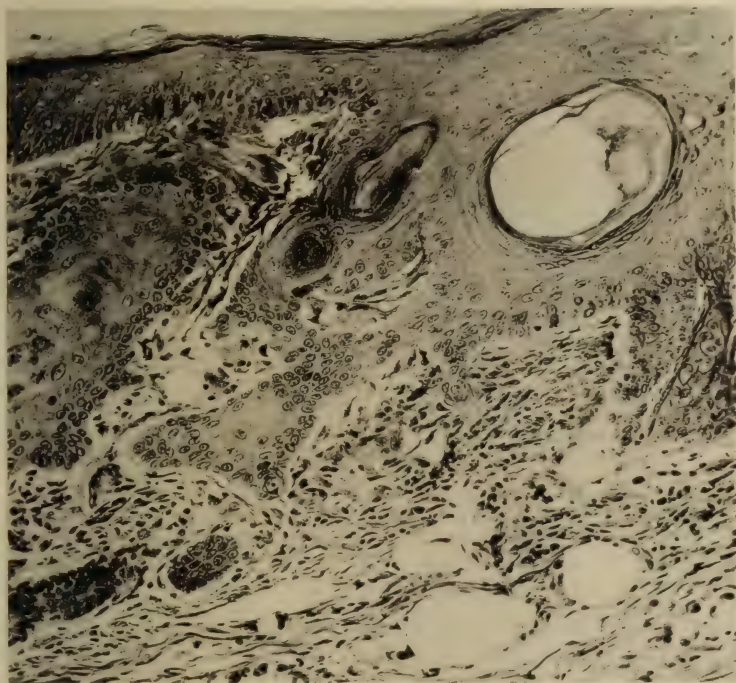


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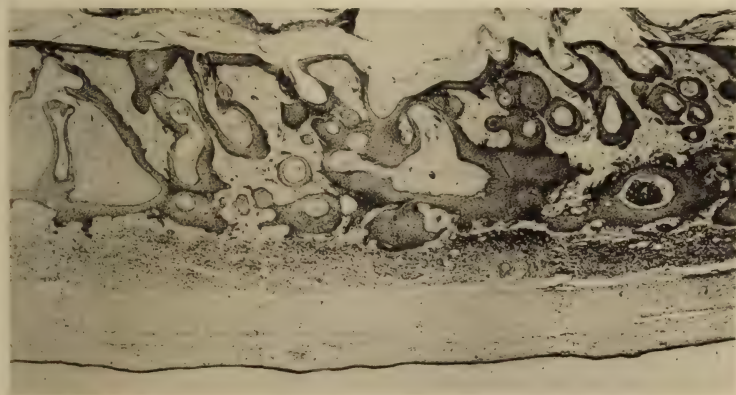
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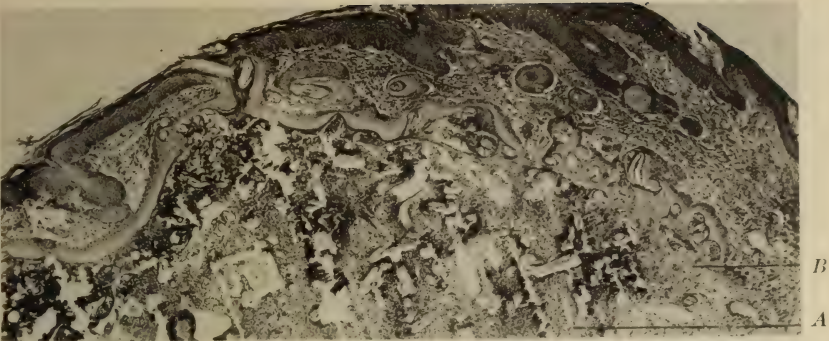


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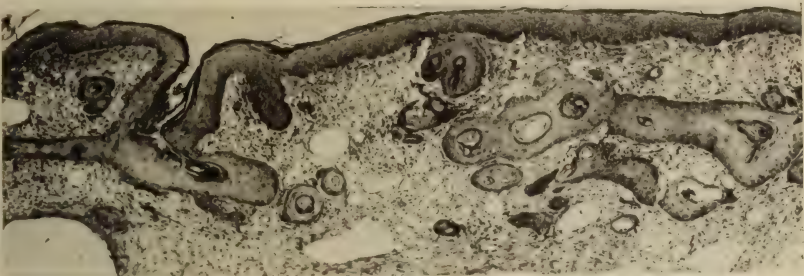


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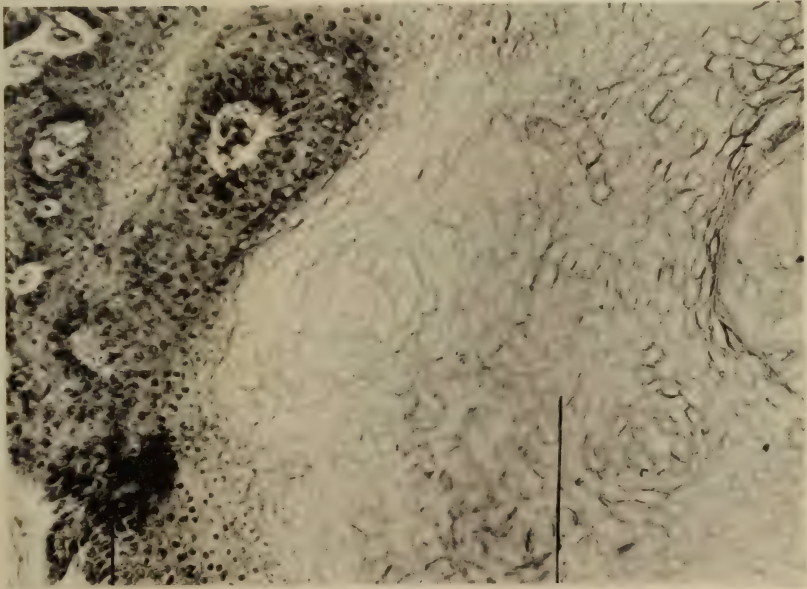
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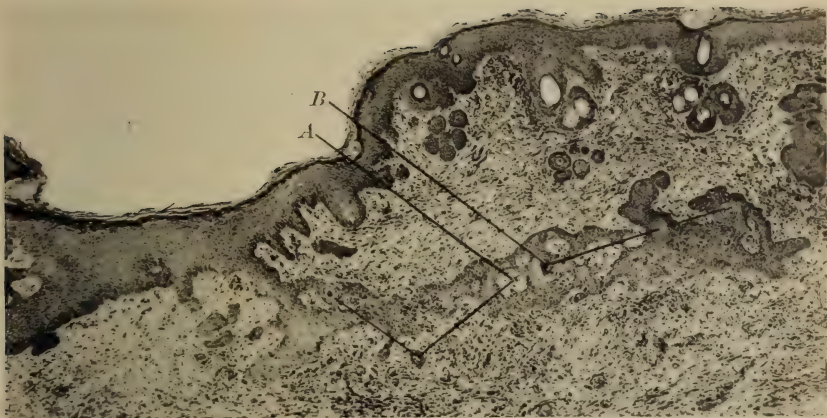


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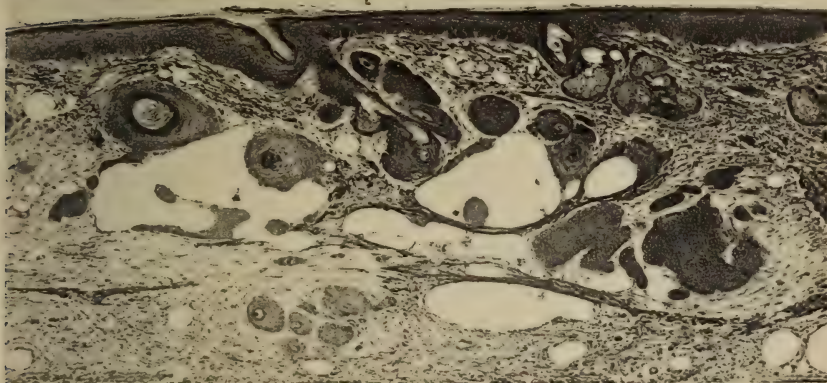
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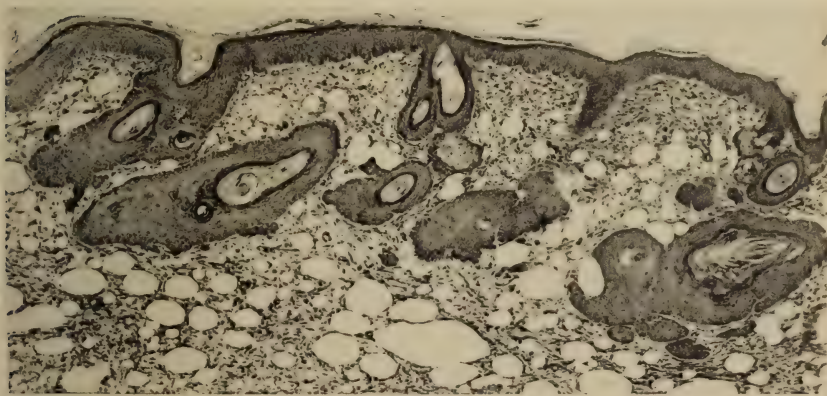
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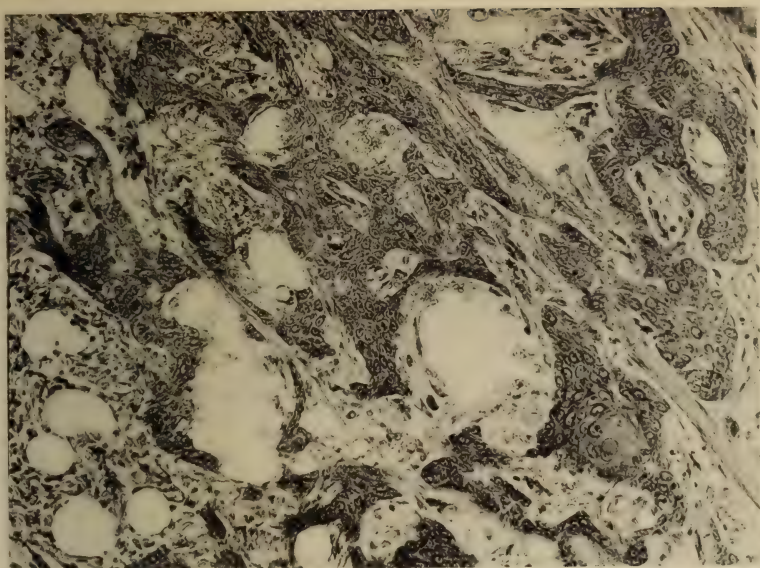


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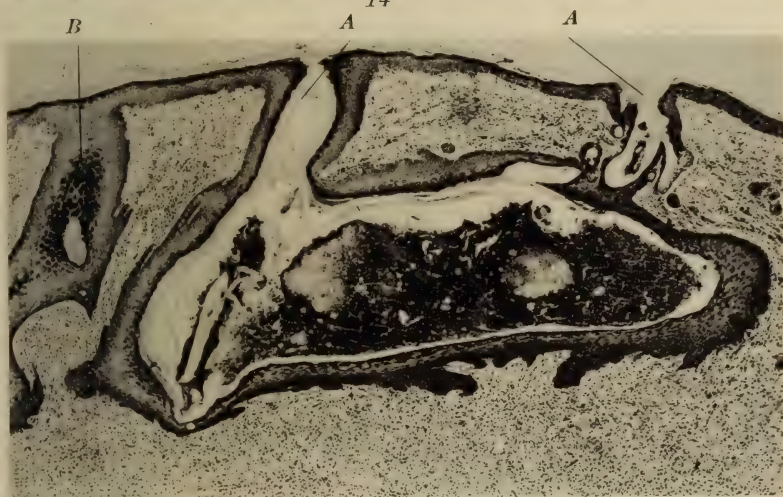


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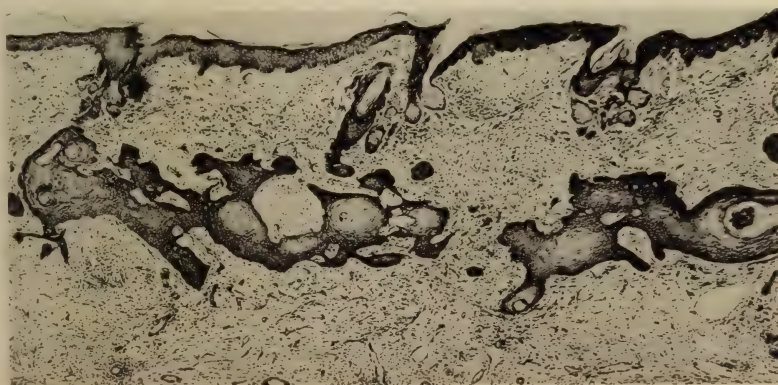
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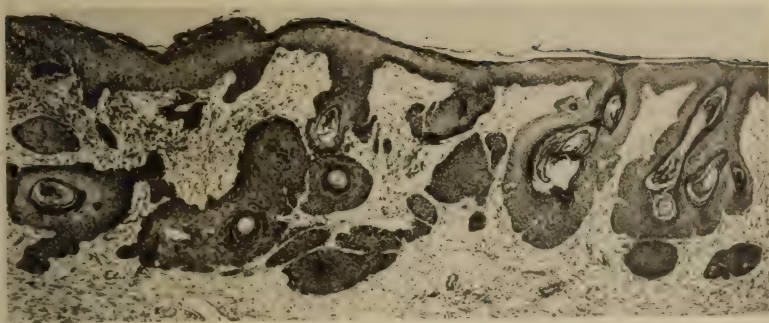


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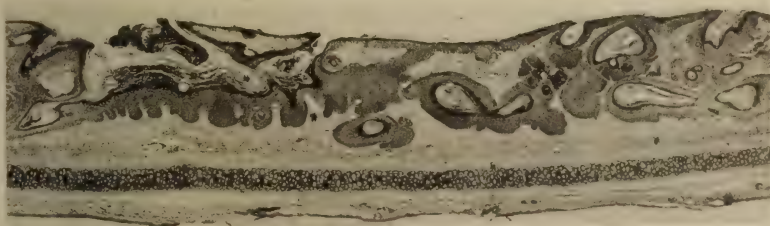


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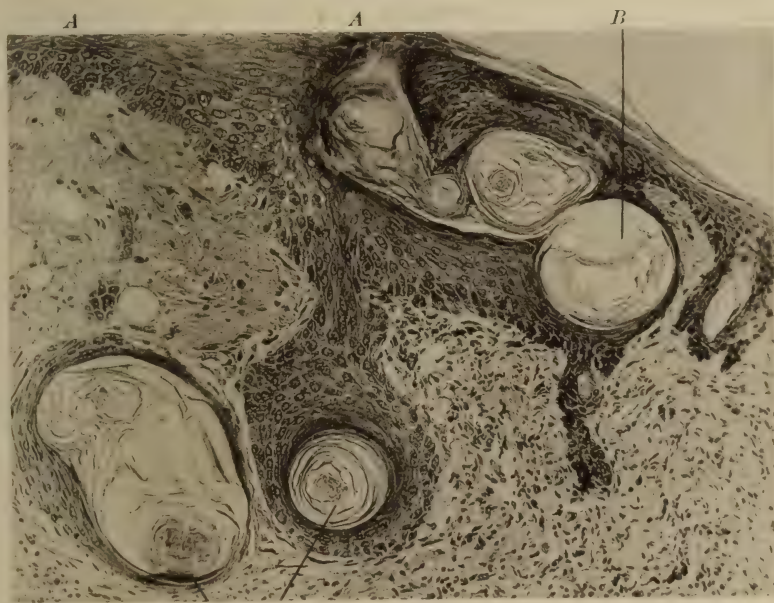
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PRIMARY MELANO-SARCOMA OF THE ADRENALS.*

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As a rule the pigmented tumors arise in the skin, choroid coat of the eye, or less frequently, in the meninges. Other organs, however, have also been mentioned as possible sources, and in the literature not a few examples of this can be found. In the works of Borst and of Ribbert on tumor, the skin, choroid, and pia mater are the only regions mentioned as primary foci. Mallory¹⁴ gives the same locations. There are, however, a number of other regions capable of giving rise to this tumor, as may be observed by the reports of Wittig, Duval, and others. Borst³ considers the esophagus, gall-bladder, and rectum as possible primary sites, while the liver, ovary, uterus, bladder, urethra, and breast have been mentioned by other authors. It is not unlikely that many of the tumors occurring in the rectum are but extensions from the pigmented area of the anal orifice, and, therefore, should be classed as having an origin from the skin. We have had a case of this type recently where the growth had extended from the anus into the rectum, producing extensive pigmented metastases. Tucek has also referred to this, but further held that the ganglionic nerve cells, which contain melanin, may be the cells from which some pigmented tumors arise and thus account for some of the very unusual situations in which this tumor is found.

Melano-sarcomata of these infrequent sources are very interesting and should be brought forward, not only on account of their rare occurrence, but also to add new facts which may have a direct bearing on the interpretation of the type of the cells which comprise them. For such reasons we are reporting a case of primary melano-sarcoma, arising in the adrenal glands. The tumor was bilateral and almost

* Received for publication May 30, 1915.

symmetrical. A complete autopsy was performed and a most exhaustive search failed to demonstrate any primary neoplasm in the skin, choroid, or meninges. The following protocol is taken from the Mercy Hospital Laboratory Records:

P. K., aged 48 years, admitted April 19, 1910, died April 20, 1910. Service of Dr. T. S. Arbuthnot. The autopsy was done one hour after death by Dr. Oskar Klotz. No history was available as the patient was comatose, and no information could be obtained from any friends. He had been picked up from the street by the police, who believed him to be intoxicated. As he lived only a few hours after admission to the hospital, a careful clinical examination was impossible. The finding of albumen in the urine, associated with the comatose condition, strongly suggested the clinical diagnosis of uremia.

AUTOPSY. — The body was that of a very tall and well-developed man whose skin surfaces had a pale, leaden appearance. Scattered in the deep subcutaneous tissues of the trunk and upper extremity were many movable nodules, averaging two centimeters in diameter. These nodules had a bluish appearance through the skin, but on section were black. None of them were attached to the epidermis. In the subcutaneous fat and recti muscles of the abdominal wall more than thirty of these black nodules were found. No mole could be found upon the skin, nor did any of the tumor masses present at the surface.

A pigmented tumor one centimeter in diameter was found in each parietal bone, and innumerable nodules were present in the brain substance and pons and medulla. A single small nodule appeared in the falx cerebri, but none were found in the leptomeninges. The eyes and optic nerves were carefully examined and found to be free from tumor. The accessory structures of the brain were normal.

In the thorax a pigmented tumor was found in a mediastinal gland and a single small mass the size of a marble was present in the right lung. The heart showed the presence of multiple small pigmented tumors in the musculature of the right auricle and ventricle and in the left auricle. A mural thrombus attached to the wall of the left ventricle was found projecting towards the aortic valve.

On opening the abdomen, the cavity was found to contain about one hundred cubic centimeters of recent blood, still in a fluid state. The abdominal viscera were everywhere coated with thin blood. Examination of the abdomen showed scattered nodules beneath the peritoneum of the abdominal wall, tissues of the mesentery, liver, bowel, and other viscera. Three large masses of tumor were also found, one on each side directly above the kidney in the region of the adrenals. Each of these masses was the size of an orange. The third mass was lying posteriorly in the

mid-line near the cardia of the stomach and attached to the left lobe of the liver. This mass was as large as a grape fruit, and its anterior surface was adherent to the stomach along the lesser curvature. On its posterior surface the mass showed a tear in its capsule and the tumor tissue was also torn. From these parts the peritoneal bleeding had its origin. Elsewhere, the tumor, which was intensely black, was covered by a thin capsule. The great omentum contained a number of nodules the size of cherries. Many of the glands in the mesentery were invaded by black growths.

Along the greater curvature of the stomach were seen two nodules raised above the general mucosal surface and having a crater-like center. This central crater was dark in color and represented tumor mass undergoing ulceration. Four small tumor ulcerations from 1 to 1.5 centimeters were seen in the duodenum. The tumor masses appeared to arise in the submucosa. Throughout the jejunum and, in fact, the whole course of the small intestine, there were innumerable (hundreds) raised pigmented nodules in the mucosa with ulcerating surfaces. Sometimes these nodules formed projecting masses like polypi with narrow pedicles. These masses were frequent in the ileum, but were also common in the cecum, some being present in the colon and sigmoid. There were two types of nodules in the intestines. The one type being shallow, crater-like ulcerating nodules which lay mainly in the submucosa. The other type formed pedunculated masses which arose from the mucosa and which hung like grapes from the mucosal wall.

Tumor masses were seen through the capsule of the liver. On section of the liver a number of encapsulated black tumor masses were observed. Several of the glands at the hilus also contained tumor tissue. The large central tumor mass, which has previously been described, measured 12 x 9 x 8 centimeters. It lay for the most part in the liver and obliterated the Spigelian lobe. This mass was very soft, black in color, somewhat lobulated and had ruptured. It was encapsulated, and rested posteriorly upon the vertebra. The gall bladder showed six small pedunculated black tumor masses at the fundus. The bile ducts were clear.

Several small encapsulated black masses, about one centimeter in diameter, were seen in the pancreas, and also in the loose connective tissue and lymph glands about the organ. A single nodule was present in the spleen.

Each kidney was of about the usual size and was surrounded by a thick layer of fat, in which were studded many small black tumors. There were no tumor masses in the kidney substance proper and both organs were normal in appearance. Over the upper pole of the left kidney and in the region of the adrenal was a large and intensely black-colored tumor mass, 10 x 8 x 4 centimeters. On section the tumor was more or less lobulated and its tissues were soft and friable. It was surrounded by a thin capsule and sharply separated from the kidney substance. No remains of adrenal substance could be found. From the renal vein, a vessel passed

over the face of the tumor and entered it at the apex. Over the right kidney, in the region of the right adrenal, was another tumor 10 x 9 x 4 centimeters. This tumor mass was similar in every way to the other on the left side. The right adrenal gland could not be recognized in the tumor mass.

A black tumor mass the size of a walnut was seen projecting into the cavity from the wall of the bladder at the fundus. The genital organs, rectum, and anus were without change.

Microscopical. Adrenal tumor mass. — There was no evidence of any normal adrenal tissue, nor did the arrangement of the tumor mass, in any way, suggest adrenal structure. The tumor was made up of a very dense cellular tissue having no special arrangement of its cells. There was a very slight stroma carrying moderately-dilated and thin-walled blood vessels. In the perivascular lymph spaces, collections of lymphoid, plasma and a few polymorphonuclear leucocytes were to be seen. The cells forming the tumor were variable in size and shape. Round, oval, and spindle forms were seen, the round forms slightly predominating. The spindle cell, at times, showed fairly long protoplasmic cell processes. Some of the round forms were small, while again others were quite large and not infrequently showed multiple nuclei. The nuclei generally were round, or oval and vesicular. The nuclei varied also in size and often appeared in multiple groups. In some cells it was impossible to make out the nucleus on account of a very large amount of coarse brown granular pigment, which more or less filled the whole cell. The pigment content of the tumor cells was very variable. Most of the cells contained some, but a few cells were quite free from it. Where the pigment was abundant, only the shape of the cell could be made out, as the protoplasm and nucleus were hidden by the heavy brown granules. Nuclear figures were fairly numerous throughout the tumor. There were no ganglionic nerve cells present, nor were there any cells which, in any way, resembled the adrenal cortical cells. The pigment did not show the presence of iron with the Nishimura test.

The tissues of the organs of the body were also examined and the tumor masses of the lungs, duodenum, spleen, and

pancreas resembled the structure as above described. The great variation in the shape and size of the cells was striking, but the difference in the quantity of the pigment also varied to a marked degree. Round, spindle, and spider-shaped cells were the most common variety found in the tumor masses of different regions, but it was not uncommon to find large syncytial pigmented masses scattered through the tumor tissue.

A careful histological examination was made of the eyes and optic nerves and nowhere was tumor found in them. Furthermore, there was an entire absence of tumor cells in the kidney, but a melanotic pigment was easily demonstrated in the cells of the convoluted tubules.

The main autopsy findings are summed up in the anatomical diagnosis. Primary bilateral melano-sarcoma of the adrenals, with secondary tumors widely distributed in all organs, save the kidneys; hemoperitoneum from rupture of tumor mass; tumorous ulceration of bowel; mural thrombus of heart.

To briefly summarize the protocol we find a primary bilateral melano-sarcoma of the adrenal with extensive metastases in a man of forty-eight years. The skin, choroid of eye, and meninges were uninvolved in tumor growth. Microscopically the tumor differed in no way from the usual melano-sarcoma which arises in the skin or in the choroid.

The bilateral nature of the tumor is, indeed, interesting from several points of view. The symmetrical appearance was striking. The tumor masses on either side were almost equal in size and shape. The shape of the tumors, roughly triangular, bore a likeness to the normal adrenal, and the relation of the tumor to the kidney was that of the normal adrenal gland. The bilateral involvement we can regard in no other light than two primary foci. This strongly suggests to us that in this case the factor of congenital misplacement of cells capable of producing melanin pigment must be considered. Both of paired organs are not infrequently involved in a similar pathological process. The congenital

cystic kidney, ovarian dermoids, and papillomatous cysts are such examples. In view of this it is not improbable that the adrenals may also at times show bilateral congenital conditions. By congenital condition in this case we do not mean to infer that the tumor itself was congenital, but in view of its cellular structure one is led to believe that previous to the tumor formation there was a congenital aberration of the pigment-containing cells, the so-called chromatophores. We have numerous examples of this in the pigmented nevi of the skin, so that it is not improbable that a congenital increase or malplacement of chromatophore cells may also occur in the adrenal, and lead later in life to the development of a malignant tumor.

Probably the most interesting finding in the case was the pale, leaden appearance of the skin associated with complete destruction of the adrenals. Why did we not have pigmentation of the skin as is seen in Addison's disease? From the man's history it is known that he was virtually a tramp and, therefore, was exposed almost continually to the open air, which would give ample opportunity for the skin to become pigmented. Lack of skin pigmentation is sometimes noted in Addison's disease, but it can usually be demonstrated by putting the patient in the sunlight to activate oxidation of the colorless pre-pigment substances. This explanation certainly does not apply to our case. The pigmentation of the skin in Addison's disease is regarded at the present time as due to a melanin pigment. An accumulation of this pigment is brought about by the destruction of the adrenal with partial or complete loss of its function. Following this there is an alteration in a series of chemical events by which there is a tendency to accumulate pigment material. The production of melanin is regarded by v. Furth as the result of two processes. By autolysis, the protein molecule is broken up and the aromatic compounds as tyrosine are acted upon by the oxidase, tyrosinase, with the development of a pigment, known as melanin. In this connection it is of interest to know that adrenalin is chemically closely related to tyrosine, and experiments (Kastle¹⁰) moreover, have

shown that adrenalin can, by the action of an oxidase, produce a pigment. Oxidizing enzymes are numerous and are present in many tissues. Meirrowsky has proved that an oxidase in the skin can by acting on adrenalin produce pigment. Similarly of great interest to us is the work of Neuberg, who isolated from a melanotic tumor of the adrenal an oxidase, which was also capable of producing a pigment by acting on adrenalin. These findings tend to show that where certain derivatives of the protein molecule come in contact with an oxidase, pigmentation is the result. Under normal conditions of metabolism this process does not occur on account of the influence exerted by the adrenal glands, particularly the medullary portion. When, however, the adrenals are interfered with, by the pathological changes in the organ, so that they do not normally carry out their functions, pigmentation develops, particularly on the exposed parts. The adrenals were totally destroyed in our case, yet there was no pigmentation; in fact, there was a very marked pallor. Total destruction of the adrenals without pigmentation of the skin has been known to occur. Sometimes the pigmentation can be demonstrated in these cases by exposing the patients to sunlight and open air. Others believe that the chromaffine cells, associated with the sympathetic ganglia elsewhere, can carry on the function of the adrenal medulla, and hence no symptoms of Addison's disease develop. Dr. Klotz has advanced an hypothesis for this odd finding. The amount of melanin pigment in the primary tumors and the metastases was very excessive. It is possible that in the production of this pigment by the tumor cells all of the essential constituents, namely the protein derivatives and the oxidases were, so to speak, used up, and were not available in the skin in a proportion sufficient to produce pigmentation. To put it briefly, in other words, the tumor cells contained all of the pigment that was produced. To say what factor was absent in the skin, whether the oxidases or the protein derivatives, we, of course, do not attempt to suggest, but it does seem highly probable that the active tumor cells contain protein derivatives in a more

available state for the action of the tyrosinase than does the skin. One point may be cited as possibly adding an argument in favor of this contention, that tumor cells are prone to undergo autolysis. In relation to this is the generally accepted theory of von Furth, of melanin production, in which he states that autolysis of the cell with liberation of the aromatic radicles of the protein molecule is the first step in the formation of the pigment melanin.

There are very few similar cases in the literature. Davidsohn⁵ refers to a case reported by him as the first primary melano-sarcoma of the adrenal with metastases that has been recognized.

The case is so like our own that we think it wise to cite the more important points. The tumor occurred in a man of 58 years. Widely distributed dark pigment growths was the principal post-mortem finding. No primary focus was found in the skin or eye. Both adrenals were enlarged, each measuring 8 x 4 centimeters. The adrenals on section still showed evidences of normal tissue, but the greater part was replaced by a large pigmented tumor mass. The tumor was made up of cells of various shapes and sizes and contained pigment in fine or coarse granules. Some cells were free from pigment. He, however, compared the tumor growth to some of the adrenal structures. Its arrangement was similar to the zona fasciculata, and moreover, the cells contained a fat-staining substance similar to that in the adrenal cells. In arrangement and form of the cells there was also a likeness to the medulla of the adrenal. Further, he added an apparently convincing fact that he had demonstrated adrenalin in the extracts of the tumor metastases.

In the discussion of the tumor Davidsohn refers to four somewhat similar cases in the literature, two in Orth's Text Book of Pathology, a case reported in the Prager Med. Wochenschr., and finally one from Marchand's Laboratory in Leipzig. All of these appear to him of doubtful adrenal origin, the first two giving insufficient proof, while Marchand¹² himself, in the discussion of Davidsohn's paper, said that the case reported from his laboratory was not clearly shown to be a primary melano-sarcoma of the adrenal, as two retrobulbar masses were present which he regarded as more likely to be the primary focus. Davidsohn concludes that his

tumor definitely arose from the adrenal, and the demonstration of adrenalin in the mesenteric lymph node metastasis was, to him, a very important point in proof of this.

Goldzieher⁶ has reported an identical case in a man twenty-nine years of age, showing a bilateral pigmented tumor of the adrenals with extensive metastases.

The adrenals measured right, 9 x 6 x 4 centimeters, left, 7 x 4.5 x 3.5 centimeters. The tumor cells were typical of melano-sarcoma. In the left adrenal there was still a considerable amount of normal-looking substance. He has very different ideas concerning the interpretation of some of the points of his case. To begin with, he regards the right adrenal as the primary site, and the left adrenal tumor merely a secondary growth. Further, he does not consider Davidsohn's observations on the presence of adrenalin correct. He was unable to confirm it in his case. Lastly, in view of Lucksch's¹¹ work on benign melanomata of the adrenal cortex, he concluded that it was from these adrenal cortical cells that his tumor arose.

Another newer case, and also one which bears a resemblance to our own, is that of Tuczek.¹⁹

We shall refer to some of the points in his well-worked-up report. The case was a bilateral primary melanoma of the adrenal without metastases in a female of 47 years. In structure the tumor was composed of variously-shaped cells containing large and small granules of pigment. Some of the cells were quite free from pigment. Tuczek enters into the question of the nature of the pigment and the origin of the cells quite thoroughly. He comes to the conclusion that the pigment in his case was a true melanin, and not a lipochrome as is normally found in the adrenal cortical cells. He therefore does not consider the adrenal cortical cells as playing any part in the origin of the tumor. There are, however, two other types of cells in the adrenal which contain melanin, and from these cells the tumor may have arisen. In the loose, connective tissue about the adrenal, both in man and in other animals, he has found chromatophore cells containing melanin, while again in the ganglionic cells of the medulla, as has been previously shown, a true melanin also is found. He leans to the second of these two possibilities, but still considers that an origin from the chromatophore cells is also very probable.

In our case the outstanding feature was that we were dealing with a typical melano-sarcoma. It differed in no way, microscopically, from similar tumors which arise in the skin or in the choroid. After an exhaustive search of the skin

and choroid as well as by ruling out the other possible sites, we concluded that the tumor was primary in the adrenal gland and bilateral in its origin.

Arising, therefore, in the adrenal, the question naturally was asked even in spite of the unusual pigmented character of the growth, whether this tumor, microscopically, would show any similarity to the hypernephromata. In this regard our findings were negative.

Lucksch,¹¹ in an article on benign melanoma of the adrenals, refers to three cases and further suggests that a close search will probably reveal many more. These tumors were small, brownish growths, lying in the cortical substance and also infringing on the medulla. They were formed by cortical cells and contained a dark pigment which Lucksch considered as melanin. All of his cases occurred in adults over fifty years. Schmorl has also spoken of small melanomata of the adrenal cortex and holds that they are by no means infrequent. Goldzieher regards these benign melanomata as having a very important role in the production later on of malignant melanotic tumors. He believes his case had such an origin.

From the medulla of the adrenal another type of tumor arises which, according to Herxheimer,⁸ can be classified in the following way: (a) neuroblastoma, (b) ganglioneuroma, (c) paraganglioma. But from the microscopic description of the cases given and from my own, there is no doubt that these tumors cannot fall under any of these classes.

In his conclusions Tuczek regards the presence of melanin pigment in the ganglionic cells as ground for the theory that this tumor may arise from altered neuroepithelium which enters into the formation of the sympathetic system in the medulla of the adrenal. Aschoff has also pointed this out in the discussion on Goldzieher's paper. Goldzieher, on the other hand, contends that too much stress must not be laid on the type of pigment as the differentiating factor of a tumor. Hueck⁹ was not able to demonstrate true melanin in the adrenal cortical cells, but adds that it is present in the

ganglionic cells. Tuzcek has confirmed these findings. The pigment in the tumor cells of our case was similar in every way to that seen in other melanomata. Ordinary stains were of no value in differentiating this pigment. The pigment did not stain with Sudan III., although in the tumor cells an occasional fine, reddish-stained granule was to be noted. This, however, is not unusual and can be demonstrated in practically any tumor. We do not, therefore, lay any stress on this finding. The Nishimura's iron reaction was negative. The pigment, therefore, as far as we can say from histological grounds, is melanin.

The important question now arises as to the origin of the tumor in the adrenal. In the first place the almost symmetrical appearance of the tumor, lying as it did in the normal position of the adrenals, cannot, we think, be regarded other than of bilateral primary origin. In shape the tumor was roughly triangular, not unlike the adrenal. We accept Ribbert's¹⁶ theory that melanomata arise from cells which have the power of producing melanin, the so-called chromatophore cells or melanoblasts. These cells, we believe, are mesoblastic in origin. Tuzcek has shown that occasional chromatophore cells are to be found in the loose connective tissue about the adrenal gland, and Aschoff¹ has also repeated this statement.

We cannot convince ourselves that the ganglionic cells played any part whatever in the tumor formation. If the tumor had arisen from these cells, it is but logical to expect on microscopic examination some suggestion of a growth of nervous origin. This was entirely wanting and we feel justified in ruling it out of consideration. The fact that ganglionic cells do contain melanin cannot be regarded as an indication that a melanotic tumor must come from that source. For like reasons we consider the adrenal cortical cells as unassociated with our tumor tissue. The tumor bore no resemblance to the adrenal cortical cells, either in appearance or arrangement. Further, our tumor was pigmented by melanin, and according to Hueck, Tuzcek, and our own observations, this pigment does not occur in the adrenal

cortical cells. The pigment in the adrenal cortical cells is a lipochrome and not a melanin.

The only cellular origin left is the chromatophore, and it is from this type of cell, we believe, the tumor arose. Tuczec has found chromatophore cells in the loose connective tissue about the adrenals in normal cases of man and other animals. After searching many adrenals, I have been unable to satisfy myself of such cells, either in the tissues about the adrenal or in the adrenal itself. However, we know that chromatophore cells are not at all times pigmented, and, therefore, might be easily overlooked. Broniatowski⁴ has shown this in his studies on the chromatophore cells of the pia mater. Ribbert¹⁶ also refers to the same fact in his writings on chromatophore cells. The negative findings, therefore, in the study of adrenals, do not, by any means, influence our opinion that these cells are not present, and by overgrowth may produce a very malignant tumor. According to Tuczec the chromatophore cells arise from the neuroepithelium. We, however, agree with those who believe that they are of the mesoblastic origin.

This question of origin of chromatophore cells we do not wish to discuss at any length, as it has been taken up more fully in the papers referring especially to that point. There are, however, several types of pigmented tumors involving the nervous system which show, when taken collectively, a common factor in their construction, namely the existence of an undue development of chromatophore cells. In the first group are the cases reported by Oberndorfer,¹⁵ Grahl,⁷ and myself,¹³ where there was a diffuse pigmentation of the skin and central nervous system in infants. The skin lesions were simple nevi pigmentosi, while the meninges and brain showed a diffuse increase of pigmented and non-pigmented nevus cells. The conditions were of congenital origin and were essentially benign. Grahl and Oberndorfer regarded the epidermis and neuroepithelium as the tissue from which the pigmented tissues arose. I held they were mesoblastic in origin. In the second group are the melano-sarcomato, arising from the chromatophore cells in the pia mater. Cases

are reported by Schopper,¹⁸ Sternberg, and others. In the last group we may class the melanomata of the adrenal which we have referred to in this paper. We think Davidsohn, Goldzieher, Tuczek, and our own case may be grouped in one class. We believe that they arose from chromatophore cells lying in the medulla of the adrenal. As we have previously mentioned, we have been unable to demonstrate typical chromatophore cells in the normal adrenal gland, still, this is by no means a conclusive argument against their presence, and in some cases, producing malignant tumors. Moreover, Tuczek has demonstrated chromatophore cells in the loose tissue about the normal adrenal, although he does not regard these as the cells from which his tumor arose. Here again the origin of the chromatophore cells from neuroepithelium comes up, to which Tuczek attributes his tumor. We, however, cannot confirm his evidence. Davidsohn and Goldzieher both held the cortical cells responsible for the tumor growth. In view of the description of their tumors we cannot believe other than that they were identical with our own, and arose from the same type of cell.

CONCLUSIONS.

1. Primary bilateral melano-sarcoma of the adrenal gland may occasionally occur.
2. Its origin in the adrenal is from chromatophore cells, which are probably congenitally aberrant.
3. The pigment of the tumor, which is melanin, differs from the normal pigment of the adrenal cortical cells, which is a lipochrome.
4. Total destruction of the adrenals by a pigmented malignant tumor may occur without any pigmentation being present in the skin.

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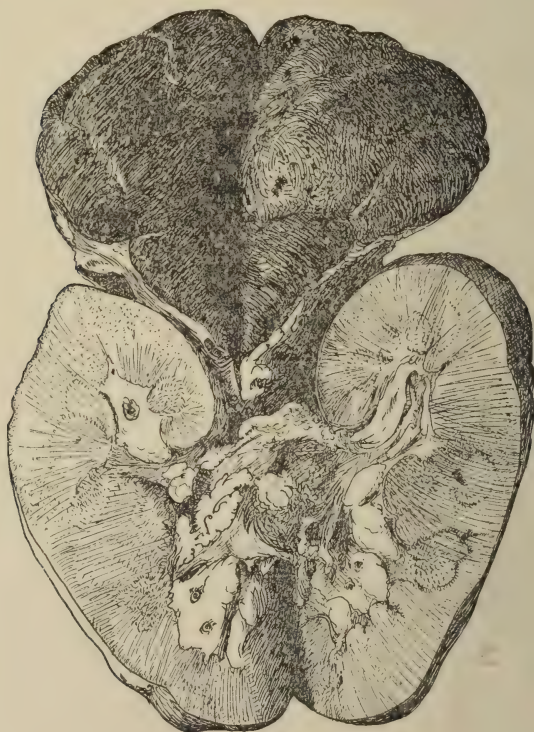


FIG. 1. — Primary melano-sarcoma of adrenal.

CONCERNING THE FILTERABILITY OF TRYPANOSOMES.*

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Novy and MacNeal,¹ in 1906, reported that in cultures of *Trypanosoma lewisi* the smallest forms which developed would pass through Berkefeld filters. In a series of nine experiments the filtrates from three were infectious for rats. Similar experiments with cultures of *Trypanosoma brucei* gave only negative results. All of these experiments were done under high pressure (50 lbs. +) with Berkefeld filters which had been reduced in thickness by sandpapering. The exact details and manner of bacteriological control, if any, were not given.

Bruce and Bateman² evidently misinterpreted Novy's and MacNeal's conclusions and accordingly reported experiments to answer the inquiry "Have trypanosomes an ultramicroscopic stage in their life history?" Three types of experiments were performed with strains of *Trypanosoma evansi*: (1) using blood and organ juices of animals simply infected with trypanosomes, (2) using blood and organ juices of infected animals which had been treated with antimony, and (3) using blood agar cultures of trypanosomes. In all cases the infectious material was passed through Berkefeld filters, proved by tests to hold back *Micrococcus melitensis*, and susceptible test animals were inoculated with the filtrates. The results were uniformly negative and led to the conclusion that neither *Trypanosoma brucei* nor *Trypanosoma evansi* produce, in the bodies of animals or in cultures, forms that can pass through the pores of Berkefeld filters.

In 1911, Bruce and his associates³ reported other experiments to determine if filterable stages of *Trypanosoma gambiense* existed during its development in *Glossiana palpalis*. In this work the intestinal tracts of flies known to be infected were dissected out and ground in a mortar with citrate saline

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solution and the mixture filtered as in earlier experiments. The results were entirely negative.

Our experiments were begun and abandoned in 1912. In spite of the entirely negative results obtained then, the experiments were resumed this year in view of the facts that Novy's and MacNeal's statement that *Spirocheta duttoni* will pass through Berkefeld filters had been satisfactorily confirmed,⁴ and that other equally large spiral organisms are known to be filterable through Berkefeld filters.⁵

The problem is not one concerning the existence of sub-microscopic forms of trypanosomes, but deals only with the possibility of some of the smallest trypanosomes passing through the filters; which is not without warrant when the filterability of spirochetes as such is taken into consideration.

Twenty-four experiments were made. In Experiments 1, 2, 3, 4, 5, and 6 the blood and organ juices of animals infected with *Trypanosoma brucei* were used, in Experiments 10 and 11 the blood and tissue juices of animals infected with *Trypanosoma gambiense*. In Experiments 9 and 12 to 24 inclusive, cultures of *Trypanosoma lewisi* were used.

Where the blood and tissue juices of infected animals were used the procedure was to open the thorax immediately after death, to remove the heart, and collect the blood from the thorax with pipettes filled with citrate saline solution. The abdomen was then opened and portions of the liver and spleen removed, and these together with portions of the lungs and bone-marrow, were ground with sand and citrate saline solution. Finally the diluted blood was added to the contents of the mortar and the whole filtered several times through a Büchner filter, in order to remove shreds of the tissue and a part of the blood corpuscles. Repeated filtrations through a well-made Büchner filter will remove a large number of the red corpuscles so that the relative number of trypanosomes becomes increased. This preliminary filtration is necessary to avoid early clogging of the Berkefeld filter,

and it does not affect the infectiousness of the trypanosomes.

Before filtering through the Berkefeld filter a suspension of the control bacterium was added to the Büchner filtrate and a portion of the latter set aside for inoculation into control animals at the end of the experiment. For Experiments 1 to 9 inclusive new Berkefeld filters were used, for the other experiments Berkefeld "V" filters, not necessarily new, were used. *Bacillus prodigiosus* was the control bacterium in every instance but two (Experiments 10 and 11), when *Staphylococcus pyogenes citreus* was used.

In experiments using cultures the Büchner filtration was omitted. The cultures, unless otherwise stated in the protocols of the experiments, were grown on a medium based on the following composition:

Agar-agar	14 grams.
Sodium chloride.....	6 "
Dextrose.....	1.5 "
Distilled water	900 cc.
Extra strength veal infusion.....	100 "

The extra strength veal infusion is made by boiling down an infusion of five hundred grams of veal in five hundred cubic centimeters of water to one hundred cubic centimeters. After sterilization and tubing, two to three cubic centimeters of a mixture of equal parts of sterile rabbit's blood and citrate saline solution were added to four cubic centimeters of the jelly and the mixture heated at 45° C. for thirty minutes. The tubes were slanted and allowed to stand until considerable water of condensation had collected, before inoculation. This medium we believe to be superior to the Novy-MacNeal-Nicolle medium; it differs only in addition of the dextrose and veal infusion. In our experience it affords more rapid growth, and hence a greater abundance of trypanosomes, while the vitality of the cultures is not diminished.

The entire amount of filtrate collected from each experiment was injected intraperitoneally into one or more white

rats, according to the quantity. One or two control rats were injected intraperitoneally at the end of the experiment with some of the mixture set aside before filtration.

Cultures were made from the filtrates at the beginning and end of the experiments, using at least one cubic centimeter for each culture tube.

Positive pressure of twenty-four pounds was used for most of the experiments. A pressure of fifty pounds was used for Experiments 18 and 19, and filtration by gravity in Experiments 17 and 22.

It is the opinion of the writers that high pressure is not of advantage in the filtration of organisms which undoubtedly owe their filter-passing property to motility and flexibility. The results obtained with further work on the filterability of *Spirocheta elusa*⁶ indicate that pores of the finest grade of Berkefeld filter, "W," are sufficiently large to pass most bacteria were it not for the tortuosity and length of the channels to be traversed. Actively motile and flexible spiral organisms succeed in finding a route. On the whole we have found that a low pressure over a considerable duration of time is preferable to the rapid forcing through of the liquid by high pressure, when it is desired to separate spirochetes from bacteria. Rapid filtration with high pressure is the best method of securing absolutely sterile filtrates. We have succeeded, however, in separating spirochetes from bacteria at all of the pressures used in these experiments.

EXPERIMENT I. — Dec. 9, 1911: Blood and organ extracts of a guinea-pig infected with *Trypanosoma brucei*. Berkefeld N filter. Pressure of 24 pounds. *B. prodigiosus* control. No control animals kept, but living trypanosomes were found in the unfiltered residuum at the end of the experiment. Two white rats were injected, each with two cubic centimeters of the filtrate. December 11th: Growth of *B. prodigiosus* in control culture tubes. Therefore defective filter. No trypanosomes in rats. December 13th: No trypanosomes in rats. December 16th: One of the two rats inoculated with filtrate contains trypanosomes.

Result. — Passage both of *B. prodigiosus* and trypanosomes, the latter in small numbers, as one of the two animals did not become infected.

EXPERIMENT II.—Dec. 13, 1911: Blood and organ extracts of a guinea-pig infected with *Trypanosoma brucei*. Berkefeld N filter. Pressure of 24 pounds. *B. prodigiosus* control. One control white rat inoculated. Two white rats inoculated with filtrate. December 15th: Bacteriological controls negative. December 21st: Control rat heavily infected with trypanosomes. December 23d: Filtrate rats negative. Jan. 6, 1912: Filtrate rats negative.

Result.—No passage of *B. prodigiosus*. No passage of trypanosomes.

EXPERIMENT III.—Feb. 7, 1912: Blood and organ extracts of a white rat infected with *Trypanosoma brucei*. Berkefeld N filter. Pressure of 24 pounds. *B. prodigiosus* control. No control animals kept, but the unfiltered residuum at the end of the experiment contained many living trypanosomes. One guinea-pig inoculated with 6 cubic centimeters of the filtrate. February 9th: One control culture tube shows growth of *B. prodigiosus*. Therefore a defective filter. February 17th: Filtrate rats negative. March 6th: Filtrate rats negative.

Result.—Passage of *B. prodigiosus*. No passage of trypanosomes.

EXPERIMENT IV.—March 2, 1912: Blood and tissue extracts of a guinea-pig infected with *Trypanosoma brucei*. Berkefeld N filter. Pressure of 24 pounds. *B. prodigiosus* control. Two white rats inoculated for controls. Two white rats inoculated with filtrate. March 4th: Growth of *B. prodigiosus* inoculated with last portion of filtrate. That from first portion is sterile. March 9th: Both controls heavily infected with trypanosomes. Both filtrate rats negative. March 16th: One filtrate rat shows many trypanosomes. The other is still negative.

Result.—Passage of *B. prodigiosus* and trypanosomes, the latter probably in small numbers, as only one of the rats became infected after an unusually long incubation period.

EXPERIMENT V.—March 23, 1912: Blood and organ extracts of two white rats infected with *Trypanosoma brucei*. Berkefeld N filter. Pressure of 24 pounds. *B. prodigiosus* control. Two white rats inoculated for controls. Two white rats inoculated with the filtrate. March 25th: Control cultures sterile. March 30th: Filtrate rats negative. April 6th: Filtrate rats negative. April 13th: Control rats infected with trypanosomes. Filtrate rats negative.

Result — No passage of *B. prodigiosus*. No passage of trypanosomes.

EXPERIMENT VI.—April 13, 1912: Blood and organ extracts of a white rat infected with *Trypanosoma brucei*. Berkefeld N filter. Pressure of 24 pounds. *B. prodigiosus* control. Two white rats inoculated for controls. Two white rats inoculated with filtrate. April 22d: Control cultures sterile. April 24th: Filtrate rats negative. April 30th: Control rats infected with trypanosomes. May 11th: Filtrate rats negative.

Result.—No passage of *B. prodigiosus*. No passage of trypanosomes.

EXPERIMENT VII.—April 27, 1912: Blood and organ extracts of a white rat infected with *Trypanosoma lewisi*. Berkefeld N filter. Pressure of 24 pounds. *B. prodigiosus* control. No animal controls kept, but living trypanosomes found in unfiltered residuum at the end of experiment. Two white rats inoculated with filtrate. May 1st: Culture tubes sterile. May 8th: Filtrate rats negative. May 16th: One filtrate rat dead, the other negative. May 20th: Remaining filtrate rat negative.

Result.—No passage of *B. prodigiosus*. No passage of trypanosomes.

EXPERIMENT VIII.—May 18, 1912: Four cultures of *Trypanosoma lewisi* made on March 1st, on blood veal agar. Water of condensation diluted with citrate saline solution. Berkefeld N filter. Pressure of 24 pounds. *B. prodigiosus* control. Two white rats inoculated as controls. Two white rats inoculated with filtrate. May 19th: Control culture tubes sterile. May 24th: Both control rats show heavy infection with trypanosomes. One filtrate rat dead, the other negative. May 27th: Remaining filtrate rat negative. June 1st: Remaining filtrate rat negative. June 16: Remaining filtrate rat negative.

Result.—No passage of *B. prodigiosus*. No passage of trypanosomes.

EXPERIMENT IX.—June 1, 1912: Blood and organ extracts of white rat infected with *Trypanosoma lewisi*. Berkefeld N filter. Pressure of 24 pounds. *B. prodigiosus* control. One white rat inoculated for control. Two white rats inoculated with filtrate. June 3d: Control cultures sterile. June 16th: Control rat heavily infected with trypanosomes. Filtrate rats negative. July 5th: One filtrate rat dead, the other negative.

Result.—No passage of *B. prodigiosus*. No passage of trypanosomes.

EXPERIMENT X.—Sept. 20, 1912: Blood and organ extracts of a guinea-pig infected with *Trypanosoma gambiense*. Berkefeld V filter. Pressure of 24 pounds. *Staphylococcus pyogenes citreus* control. Two white rats inoculated for controls. Two white rats inoculated with filtrate. September 22d: Control cultures sterile. September 26th: Both control rats contain trypanosomes. Both filtrate rats negative. October 5th: Filtrate rats negative.

Result.—No passage of *Staphylococcus pyogenes citreus*. No passage of trypanosomes.

EXPERIMENT XI.—Oct. 5, 1912: Blood and organ extracts of a white rat infected with *Trypanosoma gambiense*. Berkefeld V filter. Pressure of 24 pounds. *Staphylococcus pyogenes citreus* control. One white rat inoculated for control. Two white rats inoculated with filtrate. October 7th: Control cultures sterile. October 14th: Control rat contains trypanosomes. Both filtrate rats negative. October 21st: Filtrate rats negative.

Result.—No passage of *Staphylococcus pyogenes citreus*. No passage of trypanosomes.

EXPERIMENT XII. — May 20, 1913. A culture of *Trypanosoma lewisi* started on March 1st, in semi-solid blood veal agar, diluted with citrate saline solution. Berkefeld V filter. Pressure of 24 pounds B. prodigiosus control. Three white rats inoculated each with 1 cubic centimeter of unfiltered residuum for controls. Three white rats inoculated with filtrate. May 26th: All three control rats infected with trypanosomes. Control cultures sterile. Filtrate rats negative. May 29th: Filtrate rats negative. June 23d: Filtrate rats negative.

Result. — No passage of B. prodigiosus. No passage of trypanosomes.

EXPERIMENT XIII. — May 30, 1913: Four cultures of *Trypanosoma lewisi*, started March 1st, in semi-solid blood veal agar, diluted with citrate saline solution. Berkefeld V filter. Pressure of B. prodigiosus control. Three white rats as controls received each 1 cubic centimeter of unfiltered residuum. Three white rats injected with the filtrate. May 31st: Control cultures sterile. June 21st: Control cultures sterile. Control rats infected with trypanosomes. Filtrate rats negative.

Result. — No passage of B. prodigiosus. No passage of trypanosomes.

EXPERIMENT XIV. — Jan. 20 1915: Seven cultures of *Trypanosoma lewisi* in "N N N" medium plus dextrose, started Dec. 29, 1914, diluted with citrate saline solution to 40 cubic centimeters. Berkefeld V filter. Pressure of 24 pounds. B. prodigiosus control. Two white rats injected each with 1 cubic centimeter for controls. Two white rats injected, each with 5 cubic centimeters of filtrate. January 21st: One control rat dead. Control cultures sterile. January 22d: Control cultures sterile. January 24th: Control rat heavily infected with trypanosomes. Filtrate rats negative. January 27th: Filtrate rats negative. March 11th: Filtrate rats negative.

Result. — No passage of B. prodigiosus. No passage of trypanosomes.

EXPERIMENT XV. — Jan. 29, 1915: Nine cultures of *Trypanosoma lewisi* in "N N N" medium plus dextrose, started January 6th, diluted with citrate saline solution to 30 cubic centimeters. Berkefeld V filter. Pressure of 24 pounds B. prodigiosus control. Two white rats injected, each with 2 cubic centimeters for controls. Two white rats injected, each with 7 cubic centimeters of filtrate. February 1st: Control cultures sterile. The control rats show a few trypanosomes. Filtrate rats negative. February 3d: Control rats heavily infected with trypanosomes. Filtrate rats negative. February 5th: Filtrate rats negative. February 9th: Filtrate rats negative.

Result. — No passage of B. prodigiosus. No passage of trypanosomes.

EXPERIMENT XVI. — Feb. 1, 1915: Cultures of *Trypanosoma lewisi* in veal broth dextrose blood agar, started January 14th, diluted with citrate saline solution to 30 cubic centimeters. Berkefeld V filter. Pressure of 24 pounds B. prodigiosus control. Two white rats injected, each with 1.5

cubic centimeters for controls. Two white rats injected, each with 4 cubic centimeters of filtrate. February 3d: Control cultures sterile. Control rats show a rare trypanosome. Filtrate rats negative. February 6th: Control rats heavily infected with trypanosomes. Filtrate rats negative. February 9th: Filtrate rats negative.

Result. — No passage of *B. prodigiosus*. No passage of trypanosomes.

EXPERIMENT XVII. — Feb. 5, 1915: Ten cultures of *Trypanosoma lewisi* in veal broth agar, started January 21st, diluted to 50 cubic centimeters. Twenty cubic centimeters of this used for this experiment. Berkefeld V filter. Pressure of 24 pounds *B. prodigiosus* control. Two white rats injected, each with 1 cubic centimeter for control. One white rat injected with 4 cubic centimeters of filtrate. February 10th: Control cultures sterile. Control rats contain trypanosomes. Filtrate rats negative. February 12th: Filtrate rats negative. February 14th: Filtrate rats negative.

Result. — No passage of *B. prodigiosus*. No passage of trypanosomes.

EXPERIMENT XVIII. — Feb. 5, 1915: Thirty cubic centimeters of dilution of *Trypanosoma lewisi* cultures prepared in Experiment XVII. Berkefeld V filter, by gravity. Time, 4½ hours. *B. prodigiosus* control. One white rat injected with 2 cubic centimeters of unfiltered residuum at end of experiment for control. Two white rats injected, each with 10 cubic centimeters of filtrate. February 7th: Control cultures sterile. February 11th: Control rats heavily infected. Filtrate rats negative. February 14th: Filtrate rats negative.

Result. — No passage of *B. prodigiosus*. No passage of trypanosomes.

EXPERIMENT XIX. — Feb. 12, 1915: Cultures of *Trypanosoma lewisi* in veal broth dextrose blood agar, diluted with citrate saline solution to 38 cubic centimeters. Berkefeld V filter. Pressure of 50 pounds. *B. prodigiosus* control. Two white rats, each injected with 2 cubic centimeters for controls. Three white rats, each injected with 8 cubic centimeters of the filtrate. February 17th: Control cultures sterile. Control rats all infected with trypanosomes. Filtrate rats negative. February 16th: Filtrate rats negative. February 18th: Filtrate rats negative. February 22d: Filtrate rats negative.

Result. — No passage of *B. prodigiosus*. No passage of trypanosomes.

EXPERIMENT XX. — Feb. 20, 1915: Ten cultures of *Trypanosoma lewisi*, started February 7th in veal broth dextrose blood agar, diluted to 57 cubic centimeters with citrate saline solution. Berkefeld V filter. Pressure of 50 pounds. *B. prodigiosus* control. Two white rats injected, each with 2 cubic centimeters for controls. Four large white rats injected with the filtrate, in amount 46 cubic centimeters. February 23d: Control cultures sterile. Control rats contain a few trypanosomes. Filtrate

rats negative. February 26th: Control rats heavily infected with trypanosomes. Filtrate rats negative. February 28th: Filtrate rats negative. March 1st: Filtrate rats negative.

Result. — No passage of *B. prodigiosus*. No passage of trypanosomes.

EXPERIMENT XXI. — March 4, 1915: Ten cultures of *Trypanosoma lewisi* in veal broth dextrose blood agar, started February 17th, diluted with citrate saline solution to 50 cubic centimeters. *B. prodigiosus* control. Berkefeld V filter. Pressure of 24 pounds. One white rat inoculated with 5 cubic centimeters for control. Two white rats inoculated, each with 10 cubic centimeters of filtrate. March 8th: Control cultures sterile. Control rats heavily infected with trypanosomes. Filtrate rats negative. March 10th: Filtrate rats negative. March 14th: Filtrate rats negative. March 18th: Filtrate rats negative.

Result. — No passage of *B. prodigiosus*. No passage of trypanosomes.

EXPERIMENT XXII. — March 4, 1915: Four cultures of *Trypanosoma lewisi* started February 17th, in veal broth dextrose blood agar, diluted to 30 cubic centimeters. Berkefeld V filter, by gravity, duration 3 hours. *B. prodigiosus* control. One white rat injected with 5 cubic centimeters as control. Two white rats injected, each with 10 cubic centimeters of filtrate. March 5th: Control cultures sterile. March 7th: Control rat shows a rare trypanosome. Filtrate rats negative. March 10th: Control rat heavily infected. Filtrate rats negative. March 14th: Filtrate rats negative.

Result. — No passage of *B. prodigiosus*. No passage of trypanosomes.

EXPERIMENT XXIII. — March 9, 1915: Nine cultures of *Trypanosoma lewisi* in veal broth dextrose blood agar, started February 24th, diluted to 60 cubic centimeters with citrate saline solution, 30 cubic centimeters used for this experiment. Berkefeld V filter. Pressure of 24 pounds. *B. prodigiosus* control. Two white rats inoculated, each with 1 cubic centimeter of mixture for controls. Two white rats inoculated, each with 8 cubic centimeters of filtrate. March 13th: Control sterile. Control rats negative. March 16th: Filtrate rats negative. One control rat infected. March 18th: Filtrate rats negative. Control rats heavily infected. March 22d: Filtrate rats negative.

Result. — No passage of *B. prodigiosus*. No passage of trypanosomes.

EXPERIMENT XXIV. — March 9, 1915: Thirty cubic centimeters of the material from Experiment XXIII. used, representing one-half of the dilution of nine cultures of *Trypanosoma lewisi*. Berkefeld V filter, by gravity, duration 3½ hours. *B. prodigiosus* control. Two white rats injected for controls, each with 1 cubic centimeter of mixture. Two white rats injected with 8 cubic centimeters of filtrate. March 13th: control cultures sterile. Control rats negative. March 16th: One

control rat is infected. Filtrate rats negative. March 18th: Filtrate rats negative. Both control rats infected. March 20th: Filtrate rats negative.

Result.—No passage of *B. prodigiosus*. No passage of trypanosomes.

In this series of experiments no attempt was made to repeat exactly the conditions meagerly described by Novy and MacNeal. Our purpose has been to determine whether or no trypanosomes are filterable in the sense that certain minute bacteria and some spirochetes are filterable. The property of filterability, as stated by Novy and MacNeal, and according to work of our own,⁵ is not dependent upon minute sizes, as assumed by Bruce and Bateman.² Since all grades of Berkefeld filters allow the passage of filterable spiral organisms, and the coarsest grade the passage of small bacilli, it does not seem possible to credit trypanosomes with the property of passing filters unless they, too, will pass through the unmodified Berkefeld "V" filter. The positive results of Novy and MacNeal in the absence of contrary evidence we attribute to the thinning down of the filters used by them, and it is not possible to believe that these filters would have yielded bacteria free filtrates under the conditions of our experiments.

It is true that we used high pressure, fifty pounds or more, in but a few experiments, and then only to duplicate the conditions of Novy and MacNeal, as far as possible. Our experience in the filtration of *Spirocheta elusa* shows that for this organism low pressure, even gravity alone, and long duration of filtration are the most favorable conditions for passage through the filter. Motility and pliability seem to be the factors which are most important in the passage of organisms through Berkefeld filters. That the cross-section of the organisms must be less than the diameter of the largest pores of the filter cannot be refuted.

We have not seen trypanosomes, even in the most actively growing cultures, whose least dimension does not exceed in size the diameters of the filter-passing organisms we have worked with. It is also probable that surface tension

phenomena play a greater part holding back very plastic organisms, such as trypanosomes, than is the case with the more rigid-walled yet flexible spirochetes.

Our conclusion is that trypanosomes from cultures and from animal tissues are not filterable through bacteria-proof filters.

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THE CHOLESTEROL CONTENT OF CEREBROSPINAL FLUID.*

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In 1909 Pighini¹ reported on the cholesterol content of the cerebrospinal fluid. He extracted by Ritter's method twenty-five cubic centimeters of spinal fluid from cases of general paralysis of the insane, epilepsy, dementia precox, manic depressive insanity, pellagra, apopleptic dementia and alcoholism, as well as from normal cases. His results are expressed as no crystals, few crystals, sharp cholesterol reaction, etc. The normal, manic-depressive, pellagra, apopleptic dementia and alcoholism cases showed no cholesterol present, while the dementia precox, epileptic and parietic cases, showed amounts varying from none to a sharp cholesterol reaction.

With twenty-five cubic centimeters of fluid, a small amount of cholesterol would not be detected by the method used. Corper² showed that by Ritter's method of extraction there is an error that "may vary from five to twenty per cent in the case of normal tissue." Hepburn³ studied the various methods of determining cholesterol quantitatively, and found that the digitonin method of Windaus was the most accurate. Weston⁴ showed that in the case of serum, to which known amounts of cholesterol had been added, an excess of 8.56 per cent was recovered by the digitonin method, and an excess of .56 per cent by the method of Weston and Kent.⁵

The latter method was used in the determinations reported in this paper. In order to secure larger amounts, the fluid was obtained from cadavers immediately after death, the time varying from fifteen minutes to three hours. The cadaver was placed in a sitting posture and the needle introduced between the fifth lumbar and first sacral vertebræ. The clear fluid was collected in a beaker. When the flow

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lessened, the head of the cadaver was flexed on the thorax, so that air rushed into the needle and replaced the fluid which had been drawn off. When the head was extended, the fluid again flowed freely for a time. The flexion and extension were continued until no more fluid was obtainable. The quantity of fluid obtained varied considerably, as will be seen from the accompanying tables.

Technic of examination. — The fluid was divided into two or three portions; placed in wide-mouthed bottles and evaporated almost to dryness in an oven the temperature of which was kept at 60° C. The residue after evaporation was covered with alcohol, the quantity used being equal to, or greater than, the amount of spinal fluid represented by the residue. After twenty-four hours at 60° C. the alcohol was decanted and a second lot added to the residue. This was replaced in twenty-four hours by anhydrous ether. The alcohol and ether extracts were evaporated down to ninety or one hundred cubic centimeters, and boiled for an hour with potassium hydrate. At the end of this time the alcohol had evaporated to about twenty cubic centimeters. A saturated solution of calcium hydrate in distilled water was added, and the resulting precipitate was collected on a filter, washed, dried, and extracted with anhydrous ether. The ether was evaporated and the residue extracted with chloroform. The chloroform extract was placed in small, flat-bottomed test-tubes and evaporated to dryness. The dried extract was used for the cholesterol determinations.

Nine test-tubes, ten by one hundred millimeters and of equal bore, were set in a rack, and to each was added one cubic centimeter of a chloroform solution of cholesterol, so that the first tube contained .0001 gram and the last .0003 gram of cholesterol, each tube containing .000025 gram more than the preceding one.

Chloroform was added to the dried extracts of spinal fluid in the proportion of one cubic centimeter to each one hundred cubic centimeters of spinal fluid represented by the extract. Quantities of the dissolved extract representing

twenty, forty or sixty cubic centimeters of the spinal fluid were placed in tubes similar to those described, and enough chloroform was added to make one cubic centimeter in each tube. Several tubes were prepared from each fluid. Thus if the extract represented one hundred cubic centimeters of fluid, two tubes were prepared, one representing forty, and the other sixty cubic centimeters. In all cases at least two, and sometimes five, different quantities were tested.

To each tube of the extract and of pure cholesterol was added one-tenth cubic centimeter of pure sulphuric acid, and the tube was shaken. At the end of thirty minutes one cubic centimeter of pure chloroform was added to each tube and all were placed in the dark for fifteen minutes, at the end of which time the tubes containing pure cholesterol showed a gradation of color from a barely perceptible pink to a deep pink. The colors of the unknown solutions were then compared with those of the known, and the amount of cholesterol in each was estimated.

In some preliminary experiments it was found that the quantity of cholesterol present in the sample taken was less than .0001 gram. To determine these smaller quantities four tubes containing respectively .000075, .00005, .000025, and 0 gram cholesterol, in one cubic centimeter of chloroform, were set up, and one-tenth cubic centimeter of pure sulphuric acid was added to each. The tubes were shaken, and then one-tenth cubic centimeter of acetic acid anhydride was added to each, and the tubes were again shaken. A very pale green color developed in the tubes containing cholesterol. The tubes containing extract of spinal fluid were treated in the same manner. The colors of the extracts were then compared with those of the known quantities of cholesterol, and the amount of cholesterol in the extracts was estimated.

By the above methods thirty unknown solutions could be compared with the known ones in a few minutes. A few comparisons were made using the Autenrieth-Koenigsberger

colorimeter.⁶ This instrument was of value when only a few tests were to be made; but when the specimens to be examined numbered from thirty to ninety, so much time was required that it could not be used to advantage.

In all eighty-five fluids were examined: paresis, 26; senile dementia, 9; organic dementia based on arteriosclerosis, 9; manic depressive psychoses, 14; dementia precox, 8; epileptic psychoses, 14; cerebrospinal syphilis, 2; imbecility, 1; paralysis agitans, 1; carcinoma of brain, secondary, 1; tetanus, 1; tubercular meningitis, 1; glioma, 1.

TABLE.

General paralysis of the insane.

No.	Age.	Sex.	Weight.	Time.	Quantity.	Mgs. Cholesterol per cc.	Cause of Death.
196 . .	34	F.	125 lbs.	$\frac{1}{2}$ hr.	200 cc.	.0024	G.P.I. Chronic myocarditis.
195 . .	46	M.	150 "	1 "	175 "	.005252	" Bronchopneumonia.
192 . .	41	M.	145 "	$\frac{1}{2}$ "	200 "	.00711	" "
191 . .	76	M.	155 "	$1\frac{1}{2}$ "	180 "	.001787	" Chronic myocarditis.
188 . .	40	F.	120 "	2 "	190 "	.00572	" Bronchopneumonia.
179 . .	33	M.	145 "	$1\frac{1}{2}$ "	175 "	.00163	" Chronic myocarditis.
176 . .	56	M.	110 "	2 "	120 "	.0038	" Fatty degen. myocardium.
173 . .	40	M.	115 "	2 "	140 "	.00307	" Bronchopneumonia.
172 . .	43	M.	130 "	$2\frac{1}{2}$ "	200 "	.0055	" Fatty degen. myocardium.
158 . .	55	M.	160 "	$1\frac{1}{2}$ "	210 "	.0042	" Subdural hemorrhage.
164 . .	40	M.	180 "	1 "	200 "	.0061	" Chronic myocarditis.
163 . .	42	M.	130 "	1 "	190 "	.001	" Bronchopneumonia.
139 . .	49	F.	160 "	$1\frac{1}{2}$ "	160 "	.0052	" Cerebral thrombosis.
133 . .	47	M.	180 "	2 "	180 "	.0016	" Chronic myocarditis.
132 . .	53	F.	110 "	$1\frac{1}{2}$ "	200 "	.0043	" Fatty degen. myocardium.
107 . .	31	M.	140 "	$1\frac{1}{2}$ "	150 "	.00612	" Chronic myocarditis.
104 . .	44	M.	165 "	2 "	160 "	.00416	" " "
101 . .	35	M.	185 "	1 "	170 "	.00218	" " "
114 . .	35	M.	165 "	1 "	200 "	.00136	" Bronchopneumonia.
219 . .	30	F.	110 "	$\frac{3}{4}$ "	210 "	.00212	" Pulmonary tuberculosis.

TABLE. — *Continued.**General paralysis of the insane. — Continued.*

No.	Age.	Sex.	Weight.	Time.	Quantity.	Mgs. Cholesterol per cc.	Cause of Death.
156 . .	36	F.	115 lbs.	¼ hr.	175 cc.	.0048	G.P.I. Chronic myocarditis.
105 . .	40	M.	155 "	1 "	140 "	.0051	" General peritonitis.
130 . .	36	M.	120 "	2 "	185 "	.0024	" Chronic myocarditis.
127 . .	42	M.	100 "	2½ "	190 "	.00632	" " "
126 . .	50	M.	160 "	3 "	200 "	.00186	" Fatty degen. myo- cardium.
123 . .	43	M.	110 "	2 "	150 "	.00291	" Chronic myocarditis.

Senile dementia.

150 . .	68	M.	120 lbs.	½ hr.	125 cc.	.003	Chronic myocarditis.
131 . .	74	M.	170 "	1 "	80 "	.004	Cerebral hemorrhage.
137 . .	60	M.	175 "	2 "	100 "	.0026	Chronic myocarditis.
211 . .	65	F.	180 "	2 "	125 "	.0051	" "
115 . .	74	F.	100 "	3 "	150 "	.0038	Carcinoma, face.
108 . .	63	M.	110 "	1½ "	100 "	.006	Chronic myocarditis
167 . .	88	F.	100 "	1½ "	175 "	.0029	" "
198 . .	71	F.	150 "	2 "	125 "	.0047	Lobar pneumonia.
185 . .	86	F.	148 "	3 "	160 "	.0011	Chronic myocarditis.

Organic dementia based on arteriosclerosis.

197 . .	75	M.	130 lbs.	1 hr.	125 cc.	.00583	Chronic myocarditis.
180 . .	81	M.	145 "	1½ "	200 "	.0056	" "
174 . .	70	F.	110 "	2 "	180 "	.00588	Bronchopneumonia.
162 . .	84	M.	160 "	2 "	140 "	.0044	Chronic interstitial nephri- tis.
143 . .	62	M.	120 "	1½ "	200 "	.0062	Chronic myocarditis.
134 . .	61	F.	165 "	1 "	210 "	.0051	" "
201 . .	74	M.	170 "	1½ "	160 "	.0039	" "
220 . .	64	M.	165 "	2 "	140 "	.0026	" "
146 . .	65	F.	115 "	1½ "	200 "	.0051	" "

TABLE. — *Continued.**Imbecility.*

No.	Age.	Sex.	Weight.	Time.	Quantity.	Mgs. Cholesterol per cc.	Cause of Death.
225 . .	66	F.	130 lbs.	2 hr.	120 cc.	.0052	Chronic myocarditis.

Cerebrospinal syphilis.

213 . .	55	M.	180 lbs.	2 hr.	150 cc.	.0046	Cerebrospinal syphilis.
226 . .	53	M.	150 "	2½ "	180 "	.0072	" "

Manic depressive psychoses.

166 . .	56	M.	140 lbs.	1 hr.	80 cc.	.00387	Chronic myocarditis.
153 . .	59	F.	160 "	1½ "	200 "	.00333	" "
121 . .	60	F.	190 "	2 "	150 "	.0045	Fatty degen. myocardium.
214 . .	26	F.	130 "	2 "	90 "	.00125	Bronchopneumonia.
200 . .	54	M.	168 "	1½ "	125 "	.002	Suicide by hanging.
199 . .	46	F.	110 "	2 "	160 "	.0038	Acute nephritis.
177 . .	51	F.	170 "	1½ "	180 "	.0041	Fatty degen. myocardium.
161 . .	49	M.	150 "	2 "	200 "	.0019	Bronchopneumonia.
160 . .	57	F.	130 "	1 "	150 "	.0036	Chronic myocarditis.
155 . .	52	M.	160 "	1 "	165 "	.00461	" "
140 . .	47	F.	110 "	2 "	140 "	.00238	General peritonitis.
138 . .	75	F.	140 "	1 "	125 "	.0042	Chronic myocarditis.
112 . .	62	F.	160 "	1 "	90 "	.00125	Typhoid fever.
111 . .	49	F.	115 "	1 "	160 "	.00333	Tubercular peritonitis.

Dementia precox.

182 . .	39	F.	120 lbs.	1 hr.	100 cc.	.0011	Bronchopneumonia.
145 . .	33	M.	105 "	1 "	120 "	.00375	Chronic myocarditis.
136 . .	30	M.	160 "	2 "	60 "	.00882	" "
215 . .	32	F.	115 "	2 "	90 "	.005	Pulmonary tuberculosis.
170 . .	44	M.	160 "	1 "	140 "	.005	Bronchopneumonia.
209 . .	48	M.	160 "	1 "	125 "	.0057	Chronic myocarditis.
193 . .	50	F.	150 "	2 "	160 "	.0041	Bronchopneumonia.
223 . .	63	M.	110 "	1 "	140 "	.0026	Pulmonary tuberculosis.

TABLE. — *Concluded.*

Epileptic psychoses.

No.	Age.	Sex.	Weight.	Time.	Quantity.	Mgs. Cholesterol per cc.	Cause of Death.
210 . .	47	M.	119 lbs.	1 hr.	100 cc.	.00368	Chronic myocarditis.
206 . .	21	M.	130 "	2 "	140 "	.005	Acute nephritis.
202 . .	53	F.	132 "	2 "	160 "	.0046	Chronic myocarditis.
184 . .	49	M.	140 "	2½ "	120 "	.0021	" "
183 . .	35	M.	110 "	3 "	190 "	.0057	" "
150 . .	49	F.	105 "	2 "	160 "	.0064	" "
144 . .	40	M.	113 "	1 "	160 "	.0081	" "
102 . .	46	M.	140 "	1 "	200 "	.0043	" "
204 . .	40	M.	146 "	1½ "	140 "	.0027	Bronchopneumonia.
216 . .	36	M.	140 "	2 "	100 "	.0035	Chronic myocarditis.
227 . .	33	M.	110 "	1 "	120 "	.0029	Pulmonary tuberculosis.
218 . .	55	F.	100 "	2 "	150 "	.0067	Carcinoma of pancreas.
224 . .	45	M.	130 "	2 "	130 "	.0059	Cerebral hemorrhage.
165 . .	44	M.	150 "	2 "	100 "	.0048	Bronchopneumonia.

Miscellaneous.

Diagnosis.	Age.	Time.	Quantity.	Mgs. Cholesterol per cc.	Cause of Death.
Not insane	45	1 hr.	150 cc.	.00294	Lobar pneumonia.
Glioma	61	2 "	160 "	.0046	Glioma.
Carcinoma of brain, secondary from mam- ma	45	2 "	100 "	.005	Carcinoma, generalized.
Tetanus	20	Ante- mortem.	25 "	.0095	Tetanus.
Tubercular meningitis .	22	Ante- mortem.	3 "	.200	Tubercular meningitis.
Paralysis agitans . . .	45	1½ "	180 "	.0051	Chronic myocarditis.

Note. — The column "Time" refers to the number of hours elapsing between the time of death and the time the fluid was obtained. The body weights are approximate.

SUMMARY TABLE.

Mental Diagnosis.	Average Age.	Quantity in cc.			Mgs. Cholesterol per cc.		
		Max.	Min.	Avg.	Max.	Min.	Avg.
Paresis	43	210	120	179	.0178	.001	.003965
Senile dementia	72	175	80	127	.006	.0011	.0037
Organic dementia . . .	71	210	125	173	.0062	.0026	.00496
Manic depressive . . .	54	200	80	143	.0046	.00125	.00305
Dementia precox	42	160	66	118	.0088	.0011	.00451
Cerebrospinal syphilis .	54	180	150	160	.0072	.0046	.0064
Epileptic psychoses . .	42	200	100	141	.0081	.0021	.00467

The tables indicate that neither the quantity of fluid nor the quantity of cholesterol bears any constant relation to the psychosis. It will be noted also that the individual variation for each psychosis is considerable.

The average amount of fluid found in cases of paresis and arteriosclerotic dementia was greater than in the other cases. The average amount of cholesterol for cases of epileptic psychoses, dementia precox, and organic dementia was greater than for cases of paresis, senile dementia, and manic depressive psychoses.

All fluids examined contained some cholesterol.

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THE ACTION OF BENZOL.*

I. ON THE SIGNIFICANCE OF MYELOID METAPLASIA OF THE SPLEEN.

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In the work of Selling¹ with benzol, suggested by a series of cases which occurred in the medical clinic of Barker, it was shown that the leucocyte curve in rabbits can be lowered, by the subcutaneous administration of this substance in equal parts of olive oil, to a level corresponding to almost complete disappearance of the leucocytes from the peripheral circulation, and that the leucocyte curve may afterward rise again to the normal level as the animals recover. Selling noted considerable variation in the behavior of the erythrocyte curves following the injections. In the majority of his cases there did occur a diminution in the number of erythrocytes which, however, was slight as compared with that of the leucocytes. His average counts show that at the end of eight days there has occurred a decrease in the number of erythrocytes amounting to sixteen per cent, while the decrease in the number of leucocytes amounted to ninety-two per cent. In two of his cases the erythrocyte curve remained practically constant throughout. Histological examination of the hematopoietic organs showed extensive destruction of the specific cells of these organs, and regeneration of these cells during the course of recovery.

Selling noted the occurrence of "myeloid metaplasia" in the spleen in the later regenerative stages, but suggested that such metaplasia did not appear to be sufficiently extensive to have aided much in the regeneration of the blood cells. The relationship of so-called myeloid metaplasia in the adult spleen to blood-cell regeneration has long been a disputed point, and the recent therapeutic use of splenectomy in certain of the anemias has served to add new importance to the

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question. The work of Selling suggested the possibility of using the olive oil-benzol mixture in connection with the study of this question. It would seem that, if the spleen serves an essential compensatory function in the regeneration of leucocytes and erythrocytes in the peripheral circulation, the leucocyte and erythrocyte curves, after such destruction of circulating cells and specific cells of the hematopoietic organs, would differ in splenectomized rabbits from those occurring in corresponding non-splenectomized rabbits, and that these curves would require a longer period of time for return to the normal level during the period of recovery. Again, the regenerated blood of the splenectomized as compared with the non-splenectomized rabbits might be deficient in one or more of the varieties of blood cells, or in some other way might present a different blood-cell picture. With these considerations in view three series of rabbits were inoculated subcutaneously with olive oil-benzol mixture:

Series I., Non-splenectomized rabbits; Series II., Rabbits splenectomized two days previously; Series III., Rabbits splenectomized six months previously.

MATERIALS AND METHODS.

Series I. — Five non-splenectomized rabbits were used. Two of these died the day after the second injection was given, and the curves were thus so short as to be of no value. One recovered.

Series II. — Eight rabbits splenectomized two days previously were used. Three of these showed atypical leucocyte curves and were discarded. One of the latter was probably suffering from an infection, one died thirty-six hours after the first injection, evidently as a result of gastric hemorrhage, probably resulting from operation, and in the third no explanation for the atypical curve was found at autopsy. Three recovered.

Series III. — Eight rabbits splenectomized six months previously were used. Four of these showed atypical leucocyte curves and were discarded. Autopsy showed otitis

media in two of the latter, while no explanation of the atypical curves was found in the other two. One recovered.

Adult rabbits were used. The olive oil and benzol were always mixed together just before injections were made. All of the animals were given a sufficient number of daily injections to lower the leucocyte count to about 1,000 per cubic millimeter, after which the injections were stopped and the animals were allowed to recover. The injections were given in the abdominal region, each successive injection being made in a new place. Daily leucocyte counts were made and the injections were, in large measure, controlled by these counts.

Selling gave daily injections of the olive oil-benzol mixture in the proportion of one cubic centimeter of benzol per kilo of body weight, until the leucocyte count was 200-800. Such counts he found were associated with well-marked aplasia of the marrow in most cases. With like dosage we found that the mortality was high, and it was found to be very unsafe to administer a sufficient amount to lower the leucocyte count to 200-800 per cubic millimeter with this dosage as Selling had done.

Daily leucocyte counts were made on all animals until it seemed that the leucocyte curve had permanently risen at least to a normal level, or until the death of the animal in the case of those that did not survive. All leucocyte counts were made in the forenoon. In all instances at least three daily leucocyte counts were made before the injections were begun. Some of the animals were allowed to survive for a considerable period after the leucocytes had permanently risen to a normal level. During the latter part of these later periods, the leucocyte counts were made at intervals of four to six days.

It should be borne in mind that the normal leucocyte count of rabbits is subject to considerable variation. Variations between 3,000 and 14,000 are to be regarded as within the normal limits.

Erythrocyte counts were made in the forenoon. In the early part of the work they were made daily. Later it was

thought sufficient to make such counts on alternate days, since changes in the level of the curve were relatively slow. After the leucocyte curve had risen to and remained as high as the normal level for a considerable period the intervals between the erythrocyte counts were increased to four to six days. At least two erythrocyte counts were made before the injections were begun in all cases except three, in which only one was made.

Blood smears were made each time the leucocytes were counted. The smears were stained with Wright's blood stain and filed away until the leucocyte curves were completed. In all of the series differential counts were then made in all cases. These differential counts were made at a sufficient number of stages to trace the changes in such differential counts that might be associated with changes in the level of the leucocyte curve. In making the differential counts two hundred cells were counted, except where the number of leucocytes was so small that this was practically impossible, and then one hundred cells were counted.

The animals were weighed daily until the leucocyte curve had permanently returned to a normal level. Later the weighings were made at longer intervals. Autopsies were performed on all animals with the exception of Experiment 14.13 and Experiment 14.38, these animals still being under observation. The animals which recovered were killed and the autopsies were made immediately. The autopsies on the animals which died during the course of the experiments were usually made within one hour after death. Tissues for microscopic examination were fixed in Zenker's fluid and in ten per cent formalin. The spleens of the splenectomized animals were fixed immediately in Zenker's fluid. The results of the histological examination of tissues will not be considered in this paper.

General description of blood-cell changes in all of the series. — There will be given first, a general description of the blood-cell picture, applicable to all three series of animals. Afterward, any individual differences in the various

return to a normal level. In the secondary fall the leucocyte curve in most instances reached a level nearly as low as in the primary fall, and in some instances even lower. Selling, working with non-splenectomized rabbits only, observed that there occurred in some instances a return of the leucocyte curve to the normal level without a secondary fall, while most generally there occurred a primary rise, followed by a secondary fall and a secondary rise to a normal. In our experiments, while the primary rise might reach or exceed the level existing before injections were begun, it was in each instance followed by a secondary fall. Our experience indicates that the mortality during the secondary fall is practically as great as during the primary fall. In the animals surviving the secondary fall there occurred a return of the leucocyte curve to a normal level, this secondary rise being apparently permanent. Following the secondary fall in the leucocyte curve, the curve was judged to have returned to a normal level, when after a definite rise in the curve there occurred changes which were obviously due to daily variation in the number of leucocytes. The leucocyte curves of the animals which died during the course of the experiments were essentially the same as that shown in Chart 1, up to the time of the death of the animals.

The study of the time required for regeneration of the leucocytes in the peripheral circulation following the injections, is somewhat complicated by the occurrence of the secondary fall in the leucocyte curve. For such a study it was thought advisable to tabulate, in days after the beginning of the injections, the time of beginning of the primary fall, the lowest level reached in the primary fall, the completion of the primary rise, the time of beginning of the secondary fall, the lowest level reached in the secondary fall, and the time of return to a normal level. The leucocyte curve of each surviving animal thus shows two periods of apparent regeneration: that following the primary fall and that following the secondary fall. It would seem that any essential interference with regeneration would make itself evident in one or both of these periods. However, examination of the

tables of each of the three series of animals shows that the time intervals, the dosage, and the number of doses is essentially the same in each of the three series (Table I). As far as the table shows, the periods of regeneration are actually somewhat longer in the non-splenectomized, as compared with the splenectomized animals. The larger number of survivals in Series II. would tend to compensate for the somewhat smaller dosage and lesser number of doses. The leucocyte curves in the surviving animals of this series did not reach as low a level as in the animals of Series I. and III., although the falls in the curves were sufficiently great to give opportunity to study the periods of regeneration.

TABLE I.

Time in days after the beginning of injections of olive oil-benzol mixture of various points in the leucocyte curves.

Rabbit No. Experiment.	Beginning Primary Fall.	Lowest Level Primary Fall.	Completion Primary Rise.	Beginning Secondary Fall.	Lowest Level Secondary Fall.	Return to a Normal Level.	Number of Injections.	Cubic centimeters per kilo of Mix- ture.
Series I.:								
14.26	5	6					6	1.97
14.27	1	6	10	11	13	20	4.5	2.1
14.28	1	7	10	11	12		5	2.05
Average . . .	2.3	6.3	10	11	12.5	20	5.16	2.04
Series II.:								
14.32	1	6					6	1.91
14.33	1	3	10	11	15	20	3	1.83
14.35	1	8	10	11	15		6	1.94
14.37*	2	4	7	8	12	16	3	1.49
14.38	3	5	8	11	13	21	4	1.47
Average . . .	1.6	5.2	8.7	10.5	13.7	19	4.4	1.72
Series III.:								
14.5	4	7					6	1.94
14.6	2	8					7	1.87
14.11	2	3					2	1.97
14.13	2	5	8	10	12	15	2	1.99
Average . . .	2.5	5.8	8	10	12	15	5.4	1.95

* Autopsy showed an accessory spleen, 3 millimeters in diameter.

The erythrocyte curve.—In four of the twelve animals accepted there occurred no definite fall in the erythrocyte curve during the primary fall of the leucocyte curve, while in eight there occurred a definite fall of 500,000–2,000,000 cells per cubic millimeter. The secondary fall in the leucocyte curve in general was not accompanied by a fall in the erythrocyte curve, as was the primary fall in the leucocyte curve. In the case of one of the two Series I. animals, which survived through this period of the curves, there was no fall in the erythrocyte curve during the secondary fall in the leucocyte curve. In the case of the other, the original fall continued progressively, through the secondary fall of the leucocyte curve. In the case of four Series II. animals there occurred during the secondary fall of the leucocyte curve, a continuation of the original fall in the erythrocyte curve in one, a rise in two, and no change in one. In the case of the one Series III. animal there was a rise.

Differential counts.—The differential counts seem to show, as did Selling's counts, that the main effect of the injections is on the polynuclear leucocytes. With each fall in the leucocyte count, there is a fall in the polynuclear percentage. With each rise in the level of the leucocyte curve, there is, in general, a corresponding rise in the polynuclear percentage. The differential counts at the various stages of the leucocyte curves are shown in Table II. and on Charts 1, 2, and 3. The great difficulty which we experienced in identifying the leucocytes after the injections were begun, led us to adopt a simple classification into polynuclears, mononuclears, and unclassified cells.

The weight curves.—The weight curves tended to follow the leucocyte curves in all three series, in the manner illustrated in Chart 1. This corresponds to observations in regard to the eating and activity of the animals. When the level of the leucocyte curves was low, the animals ate little or nothing, and were inactive. With both the primary and secondary rises of the leucocyte curves, the animals ate more, and became more active.

TABLE II.
Differential counts during various stages of leucocyte curves.

Series No.	Animal No. Experiment.	Before Injections.			Lowest Level Primary Fall.			Completion Primary Rise.			Lowest Level Secondary Fall.			Return to a Normal Level.		
		Leucocytes.	Polynuclear.	Mononuclear.	Unclassified.	Leucocytes.	Polynuclear.	Mononuclear.	Unclassified.	Leucocytes.	Polynuclear.	Mononuclear.	Unclassified.	Leucocytes.	Polynuclear.	Mononuclear.
I. . . .	14.26	6,480	48	51	. . .	216	33	60	7							
I. . . .	14.27	9,800	56	44	. . .	560	10	90	. . .	4,820	86	14	. . .	800	4	87
I. . . .	14.28	7,700	46	53	. . .	440 ¹	29	69	1	1,540	37	62	. . .	380 ²	14	84
II. . .	14.32	8,260	48	47	4	200	19	81								
II. . .	14.33	13,066	34	66	. . .	1,120	13	87	. . .	6,480	52	48	. . .	1,860		99
II. . .	14.35	11,460	39	58	2	800	5	94	. . .	4,100	56	43	. . .	400	5	93
II. . .	14.37	8,380	32	66	1	1,380	1	98	. . .	7,920	64	33	2	3,420	22	76
II. . .	14.38	10,740	25	74	. . .	2,100	9	91	. . .	7,840	52	42	5	2,300	16	82
III. . .	14.5	9,125	45	53	1	150	35	23	38							
III. . .	14.6	13,940	40	57	2	180	10	90	. . .	3,400	93	5	1			
III. . .	14.11	6,260	45	55	. . .	400	10	88	1							
III. . .	14.13	9,940	47	50	2	980	2	96	1	5,020	65	30	4	1,160	3	93
														6,560	31	66
																3

¹Differential count was made on preceding day when leucocyte count was 680.

²Differential count was made on preceding day when leucocyte count was 860.

There were three exceptions to this rule among the twelve accepted animals — two in Series II., Experiment 14.33 and Experiment 14.38, and one in Series III., Experiment 14.13. However, in Series II., the weight curve is complicated by the recent splenectomy. In animal Experiment 14.33 there was a rapid fall to the end of the primary fall in the leucocyte curve, and then a gradual rise. In animal Experiment 14.38 (Chart 2) there was a fall during the primary fall of the leucocyte curve, a rise at its climax, then the weight remained practically constant until the climax of the secondary fall, after which it gradually rose. In animal Experiment 14.13 (Chart 3) the weight curve fell during the primary fall of the leucocyte curve, but rose again at the climax of the fall, then gradually fell again until the climax of the secondary fall, when it gradually rose again.

Series I. Non-splenectomized rabbits. — The leucocyte curve of a non-splenectomized rabbit which received injections is shown in Chart 1, and follows closely that shown by Selling¹ in his Table III. (page 14). On account of this close correspondence it was not thought important to have a large number of animals in this series. Myeloid metaplasia of the spleen, as described by Selling,¹ was found in the one animal of this series which reached the later regenerative stages (Experiment 14.27). In the animals of this series the average duration of the period of regeneration of leucocytes in the peripheral circulation, following the primary fall, was four days; that following the secondary fall was seven and one-half days. In this series one animal died during the primary fall, one during the secondary fall, and one was killed after it seemed that there had occurred a permanent return of the leucocyte curve to a normal level at the end of the secondary rise.

Series II. Rabbits treated two days after splenectomy. — In the case of the rabbits in which injections were begun two days after splenectomy, an example of which is given in Chart 2, the leucocyte and erythrocyte curves are essentially

the same as in the non-splenectomized rabbits. Secondary falls in the leucocyte curves occurred in each instance in which the animal survived sufficiently long. With the primary rise in one rabbit, Experiment 14.38 (Chart 2), the curve reached the original normal level and remained practically at this level for six days, after which the secondary fall occurred. In the animals of this series the average duration of the period of regeneration, following the primary fall, was three and one-half days, that following the secondary fall was five and one-third days. The results of the differential counts in this series differ in no essential from those of the normal rabbits.

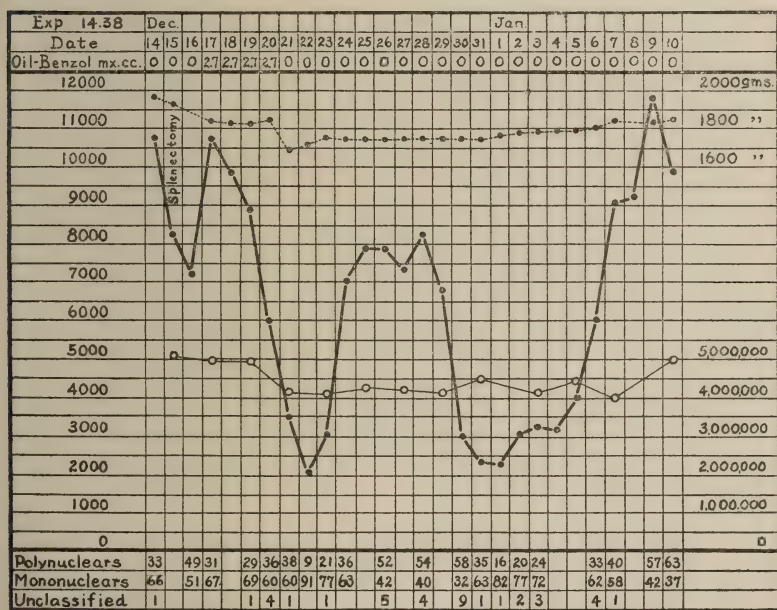


CHART 2, SERIES II. (Exp. 14.38).

Series III. Rabbits treated six months after splenectomy. — Changes in the blood of a rabbit following injections six months after splenectomy are shown in Chart 3. The leucocyte and erythrocyte curves and the differential counts are

seen to differ in no essential from those of the non-splenectomized animals, and those treated two days after splenectomy.

In the animals of this series the average duration of the period of regeneration, following the primary fall, was three and one-third days, while the duration of the period of regeneration, following the secondary fall, in the animal that survived was three days.

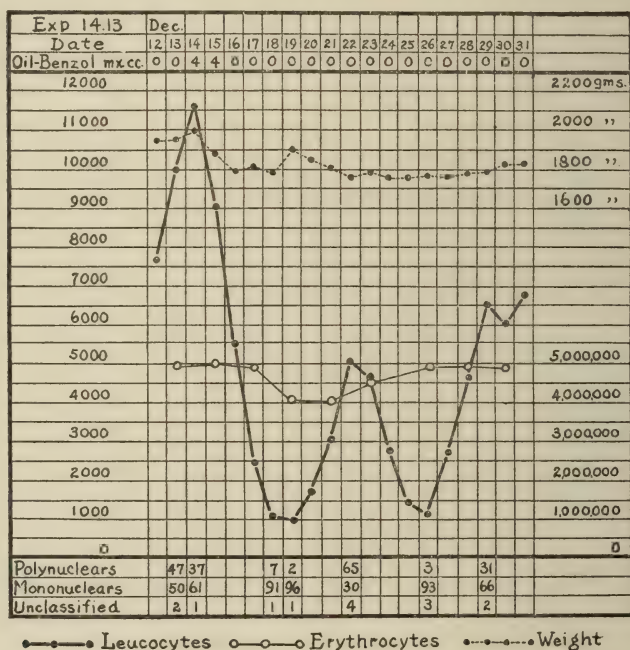


CHART 3, SERIES III. (Exp. 14.13).

Discussion. — These results confirm the results of the work of Selling in that they show the leucotoxic effect of subcutaneous injections of olive oil-benzol mixture, the polynuclear leucocytes being mainly affected. By virtue of its leucotoxic qualities it furnishes a means of destroying the leucocytes in the peripheral circulation of the living animal, and hence furnishes an unusual opportunity to study their regeneration.

Following the subcutaneous administration of the mixture in the non-splenectomized rabbit, the rabbit two days after splenectomy, and the rabbit six months after splenectomy, the leucocyte and erythrocyte curves are essentially the same. The time intervals between various parts of the leucocyte curve are essentially the same. The duration of the periods of regeneration is essentially the same in all of the series. These data seem to indicate that the spleen serves no essential function in the regeneration of blood cells in the peripheral circulation, following their destruction after the administration of the mixture. This conclusion is supported especially by the results obtained in the animals treated only two days after splenectomy. In so short an interval one could hardly expect the establishment of a compensatory function on the part of other tissues.

The secondary fall in the leucocyte curves which appears to be of unusual interest will be discussed in a later paper.

CONCLUSIONS.

1. Following the subcutaneous administration of equal parts of olive oil and benzol in rabbits, there occurs a rapid decrease in the number of leucocytes in the peripheral circulation.

2. Following this primary fall in the leucocyte curve there occurs a primary rise, which is, in each instance, followed by a secondary fall before a permanent rise to a normal level.

3. The primary and secondary falls in the leucocyte curves are accompanied by a marked decrease in the percentage of polynuclear leucocytes, indicating that the polynuclear leucocytes are especially affected after the injections.

4. Coincidentally with the primary fall in the leucocyte curve, there occurred in two-thirds of the cases a moderate but definite fall in the erythrocyte curve. The erythrocyte curve appears to progress independently of the leucocyte curve after the primary fall, and in the majority of instances remains unaffected during the secondary fall in the leucocyte

curve. This fall in the erythrocyte curve was usually followed by a rise.

5. In rabbits in which the injections are begun two days and six months respectively after splenectomy, the leucocyte and erythrocyte curves are essentially the same as in the non-splenectomized rabbits which receive like injections, and the duration of the periods of regeneration is essentially the same.

6. The differential counts on the non-splenectomized animals and those animals splenectomized two days and six months previously, show no essential differences, and the blood-cell picture is otherwise the same.

7. These observations on the leucocyte and erythrocyte curves indicate that in the rabbit the spleen serves no essential function in the destruction of leucocytes and erythrocytes, following subcutaneous injections of the olive oil-benzol mixture, or in their subsequent regeneration. Any destructive or regenerative function that it has is quickly assumed by some other part of the body when the spleen is removed. "Myeloid metaplasia" of the spleen is of no great importance as a compensatory phenomenon, and regeneration, as represented by the blood-count, takes place at least as quickly in the absence of the spleen.

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THE BLOOD-PICTURE OF HEALTHY RHESUS MONKEYS.*

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In recent years the use of the monkey as an experimental animal for the study of those infectious diseases not readily transmissible to the smaller animals has made it necessary to have accurate information as to the blood-picture of healthy monkeys. In some infections the blood-count, especially that of the white cells, is of great importance; but the information available in the literature on this subject is very unsatisfactory.

In certain studies which have been in progress at the Hygienic Laboratory during the past year it was necessary to know the normal blood-count of the rhesus monkey in order that deviations from the normal, as a result of certain experimental procedures, could be accurately gauged.

No doubt others who have used the monkey for experimental purposes have felt the need for such information, and for that reason we wish to make publication of our findings, in which are set forth the average number of red and white cells, average per cent of hemoglobin, and average per cent of leucocytic elements by differential count, as found by us in healthy *Macacus rhesus* monkeys.

Klieneberger and Carl¹ made the following observations in the varieties of monkeys cited: A species of *Macacus*, a Java monkey, a spider monkey, a bonnet monkey, and a Pavian monkey. Red blood cells (observations in 6 animals) average 6,352,000 per cubic millimeter, hemoglobin (observations in 6 animals) sixty-seven per cent. White blood cells (observations in 6 animals) 7,470 per cubic millimeter.

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The following kinds of monkeys, and some not classified, constituted the basis for further work: Two Java monkeys, a "long-tailed" monkey, a Rhesus monkey, and a Pavian:

Differential counts (26 observations in 26 animals):

Polymorphonuclear leucocytes	average	35.8%
Large lymphocytes	} Basophiles.....	" 59.3%
Small " "		
Large mononuclears		
Eosinophiles	"	3.5%
Mast cells	"	0.3%
Transitionals.....	"	1.2%

Hektoen and Eggers² state that the animals used in their experiments showed a relatively high proportion of lymphocytes. This approximates the experience of Sellards and Baetjer,³ who state the normal rates for the monkeys used by them to be: Mononuclears, sixty per cent, polynuclears forty per cent. These authors also state that a white blood-cell count of 25,000 represents about the normal for the monkey, a statement which is confirmed in a discussion of the paper by Schneisser.

The methods used in the present investigation were as follows: Ten monkeys (*M. rhesus*) were used, each about two years of age, four males, six females. White blood cells estimated for each animal every day for ten days (100 observations in 10 animals), average, 11,192 per cubic millimeter; red blood cells estimated on the first and tenth days (20 observations in 10 animals), average, 4,643,000 per cubic millimeter; hemoglobin estimated on the first and tenth days (20 observations in 10 animals), average, 75.4 per cent; differential counts estimated for each animal every day for ten days (100 observations in ten animals). As one hundred or more cells were differentiated at each observation, more than one thousand cells were observed for each animal, and a total of more than ten thousand white blood cells were observed. The detailed results are shown in the table.

BLOOD-PICTURE OF TEN NORMAL MACACUS RHESUS MONKEYS.

Monkey No.	11.	12.	13.	14.	17.	18.	19.	20.	23.	24.	Average.
Sex	Male.	Male.	Male.	Female.	Female.	Female.	Female.	Female.	Female.	Male.	
Species	M. Rhesus.	M. Rhesus.	M. Rhesus.	M. Rhesus.	M. Rhesus.	M. Rhesus.	M. Rhesus.	M. Rhesus.	M. Rhesus.	M. Rhesus.	
Probable Age	2 Years.	2 Years.	2 Years.	2 Years.	2 Years.	2 Years.	2 Years.	2 Years.	2 Years.	2 Years.	
W.B.C., per cubic millimeter	12,640	11,460	10,080	12,140	12,056	11,710	11,580	9,430	12,540	8,288	11,192
R.B.C., per cubic millimeter	4,792,000	4,764,000	4,268,000	6,068,000	4,030,000	3,600,000	4,470,000	4,696,000	5,090,000	4,661,000	4,643,300
Hemoglobin, % . . .	78.	62.5	76.	72.5	82.	75.	80.5	73.	74.	80.5	75.4
Neutrophils (polymorphonuclears), %	39.74	39.84	37.9	49.1	32.	41.2	49.6	46.3	41.1	39.3	41.61
Basophiles (lymphocytes and large mononuclears), %	57.38	59.36	60.6	49.09	61.3	54.5	37.7	49.9	47.6	57.1	53.65
Eosinophiles, %01	.1	.6	.7	4.1	3.8	11.9	3.	9.6	3.1	3.69
Mast Cells, %06	.1	.1	.1	.3	.5	.4	.2	.6	.0	.24
Unclassified, Transitional, %	2.72	.6	.8	1.01	.3	.0	.4	.6	1.1	.5	.8

In classifying the white cells we found, as did Lucas and Prizer,⁴ that an arbitrary division of the mononuclear cells was not satisfactory; so large and small lymphocytes and large mononuclears have been classed together as follows:

Neutrophiles	average	41.61%
Basophiles.....	"	53.65%
Eosinophiles	"	3.69%
Mast cells	"	.24%
Unclassified.....	"	.80%

In making the counts, blood was obtained from the ears, using the right and left alternately. Care was taken to avoid previous punctures and veins, if possible. Counts were made in the usual manner with the Thoma-Zeiss hemocytometer. The percentage of hemoglobin was estimated by the Dare hemoglobinometer. Smears were made on perfectly clean glass slides. Thirty-four were stained with Giemsa stains by the rapid method, and sixty-six with Wright's modification of Leishman's stain. Special care was taken to use only perfect fields, as deviation from this practice was found to cause wide variations in the percentage of leucocytes.

Although no precise observations were made in regard to the morphology of the red corpuscles, yet certain general characteristics were noted as follows: The red cells generally presented a normal appearance, atypical forms being rare. Some variation in size and shape was not uncommon. Monkey No. 19 presented considerable variation in the size and shape of these cells and some polychromatophilia, no stippling or blasts, Hb. = 80.5 per cent, eosinophiles, 11.9 per cent. A single examination of the stools was negative for animal parasites. One normoblast was found in the blood of Monkey No. 24, the only nucleated red cell observed during this study.

In comparing our results with those of others for monkey and human blood, the following general summary may be made:

Red blood cells. — Our findings are distinctly lower than

those of Klieneberger and Carl,¹ and slightly lower than those generally accepted for man.

Hemoglobin. — Our findings are higher than those of Klieneberger and Carl,¹ and more nearly approximate the normal values for man.

White blood cells. — Our figures are higher than those of Klieneberger and Carl;¹ but, on the other hand, are much lower than the figures given by Sellards and Baetjer and Schneisser.³ Our figures are within normal limits for man.

Differential count. — The experience of Klieneberger and Carl,¹ Hektoen and Eggers,² and of Sellards and Baetjer,³ coincides with our own in regard to the relative preponderance of basophiles over the polymorphonuclear leucocytes in monkey blood, contrary to the opposite ratio in adult human beings. Such a result, however, would not be wholly unexpected should it appear that monkeys of the same age-development as young children were used in the above observations. Under such circumstances a preponderance of the basophilic or mononuclear elements might occur, as it does in young children.

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A HISTOLOGICAL STUDY OF THE INTERNAL SECRETORY
GLANDS IN MICE BEARING SPONTANEOUS TUMORS.*

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In their search for etiologic factors in the genesis of cancer, investigators have not ignored the glands of internal secretion. Some of them have explained the formation of cancer in terms of insufficiency of certain of the internal secretions. The thyroid, thymus, ovary, adrenal, and pancreas all have been held responsible for the cancer condition, and their secretions or extracts have at one time or another been in some use as therapeutic agents in the treatment of malignant disease.

If perverted function manifests itself in histological changes, then a careful microscopical study of the endocrinous glands of spontaneous tumor-bearing mice may throw some light on the question of the participation of these glands in the cancer process. The material studied comprises one hundred mice bearing spontaneous tumors, all females, twenty-five non-tumor-bearing old females of about the same age as the tumor-bearing ones and from the same stock, fifty young females, and the thyroids and adrenals of fifty old males, both of these last being from a stock differing from the first two sets. In addition the same organs were examined in a large series (about two hundred) of non-tumor-bearing white rats. These last have not been tabulated.

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The organs examined were the liver, kidney, spleen, pancreas, ovary, and adrenals, and the thyroid, parathyroid, thymus, and pituitary glands.

By reason of the fact that the majority of the animals were killed with ether no note has been made of the congestions encountered.

Liver.—The almost invariable presence of intestinal disorders of various kinds, as well as the frequent occurrence in laboratory rodents of parasitic infections of the liver would lead one to expect various types of degeneration in this organ. These expectations were realized in all of the groups examined, both in mice and rats. The degenerations encountered were of two general types, fatty and parenchymatous; these occurred in varying degrees of severity. Occasionally focal necroses were observed, and in some instances chronic inflammation characterized by round cell infiltration; however, none of the changes were characteristic of one group of animals. The appended Table I. gives in detail the frequency of the various lesions. (Here, as in subsequent tables, it may be noted that percentages and number of animals do not always tally. The explanation of this discrepancy is found in the frequent occurrence of several lesions in the same organ.)

TABLE I.

Liver.

	Mice with Spontaneous Tumor.	Old Controls.	Young Controls.
Number examined	88	25	50
Normal	4 (4%)	0	4 (8%)
Fatty degeneration	31 (35%)	7 (28%)	30 (60%)
Parenchymatous degeneration.....	40 (45%)	18 (72%)	30 (60%)
Focal necrosis	6 (6%)	1 (4%)	
Round cell infiltration	14 (15%)	4 (16%)	10 (20%)

Kidney. — Nephritis of varying degree was a change common to all groups of animals. As in man, the lesions may be broadly grouped into three classes, one involving chiefly the parenchyma, another chiefly the stroma, and a third both parenchyma and stroma to about equal extent. A majority of the kidneys of the old mice examined showed infection with *Klossiella muris*. In the rats changes of a similar type occurred in about the same frequency, though protozoan infection was seldom observed. The numerical details are given in Table II.

TABLE II.

Kidney.

	Mice with Spontaneous Tumor.	Old Controls.	Young Controls.
Number examined.....	91	25	50
Diffuse nephritis	12 (13%)	5 (20%)	5 (10%)
Parenchymatous nephritis	72 (78%)	11 (44%)	40 (80%)
Interstitial nephritis	7 (7%)	7 (28%)	
Normal.....	0	2 (8%)	5 (10%)

Spleen. — Four types of pathological conditions were encountered in the spleen: a lymphoid hyperplasia, a moderate grade of fibrosis with perisplenitis, an hyperplasia of the endothelial elements of the organ, and amyloid degeneration. With the exception of amyloid degeneration these changes occurred in approximately the same degree of frequency in all groups, and, like the lesions noted in the liver and kidney, were also common to the rat. Amyloid disease, however, occurred only in the tumor-bearing group. The numerical details are given in Table III.

TABLE III.

Spleen.

	Mice with Spontaneous Tumor.	Old Controls.	Young Controls.
Number examined.....	82	23	48
Normal.....	16 (19%)	12 (52%)	15 (31%)
Fibrosis	13 (15%)	3 (13%)	7 (14%)
Endothelial hyperplasia	7 (8%)	1 (4%)	
Lymphoid hyperplasia.	23 (28%)	7 (30%)	26 (54%)
Amyloid degeneration	6 (7%)	0	

Pancreas. — No distinctive pathological changes could be demonstrated in the pancreas of the tumor-bearing group. Lesions of the pancreas were not very frequent. The most common were fatty degeneration and necrosis usually accompanied with fibrous changes of moderate degree. The areas of necrosis bore no relation to the islands of Langerhans. Pancreatic disease was more common in rats than in mice. The exact percentages are given in Table IV.

TABLE IV.

Pancreas.

	Mice with Spontaneous Tumor.	Old Controls.	Young Controls.
Number examined.....	64	19	44
Normal.....	36 (56%)	13 (68%)	39 (88%)
Fibrosis	8 (12%)	4 (21%)	5 (12%)
Necrosis	7 (10%)	2 (10%)	
Fatty degeneration	14 (21%)	0	
Round cell infiltration	2 (3%)	0	

Ovary. — In examining the ovary, sexual function as well as pathological changes were considered, the criterion of procreative activity being the presence of healthy Graffian follicles. The old and young controls presented a larger percentage of normal and sexually functioning organs than did the tumor-bearing group. Since sexual function depends upon the age of the animal this was to be expected, inasmuch as the tumor-bearing group consisted solely of old females. No lesion characteristic of tumor-bearing animals was observed; the older animals, however, showed a greater percentage of fibrous and fatty changes. Table V. gives in detail the various types of degeneration encountered.

TABLE V.

Ovary.

	Mice with Spontaneous Tumor.	Old Controls.	Young Controls.
Number examined	80	24	48
Normal	43 (53%)	15 (75%)	45 (94%)
Functionating	43 (53%)	20 (83%)	46 (96%)
Not functionating.....	33 (41%)	2 (8%)	2 (4%)
Function questionable	4 (5%)	2 (8%)	
Hyaline changes.....	2 (2%)		
Fatty changes	7 (8%)		
Cystic changes	4 (5%)		
Fibrosis	24 (30%)	5 (20%)	3 (6%)

Thymus. — Aside from regressive changes no lesions were noted in the thymus. Atrophy was found most frequently in the tumor-bearing and old control groups. The numerical details are given in Table VI.

TABLE VI.

Thymus.

	Mice with Spontaneous Tumor.	Old Controls.	Young Controls.
Number examined	82	23	45
Atrophic.....	71 (86%)	9 (39%)	3 (6%)
Persistent	11 (13%)	14 (60%)	42 (94%)

Pituitary. — The pituitary gland, which exerts so marked an influence on growth and development, was the one organ practically normal in both control and tumor-bearing groups, but one diseased gland being found in the thirty-five examined. The details of the examination are given in Table VII.

TABLE VII.

Pituitary.

	Mice with Spontaneous Tumor.	Old Controls.	Young Controls.
Number examined	20	15	
Normal.....	19 (95%)	15 (100%)	
Round cell infiltration	1 (5%)	0	

Parathyroid. — The predominating lesion in the parathyroid gland in the tumor-bearing animals was fibrosis. The same change was encountered in a smaller percentage of the old controls, but was practically absent in the young control animals. The exact figures are given in Table VIII.

TABLE VIII.

Parathyroid.

	Mice with Spontaneous Tumor.	Old Controls.	Young Controls.
Number examined	60	18	30
Normal.	33 (54%)	13 (72%)	28 (94%)
Fibrosis	25 (41%)	5 (27%)	2 (6%)
Round cell infiltration	2 (3%)	0	

Thyroid. — The most interesting of the various pathological pictures were seen in the thyroid gland. In the gross, the glands were hypertrophied in many of the animals of the tumor-bearing group and the old female controls. The lesions encountered were of a progressive nature and consisted of three general types of hypertrophy, an exophthalmic type, a colloid type and a cystadenomatous type (see Figures 1, 2, 3). Papillary cystadenomata of varying size, some easily demonstrable in the gross, were of very frequent occurrence. Fibrosis was less frequent. In rats, acinar hypertrophy is fairly common though papillary cystadenomata are extremely rare. Table IX. gives the numerical data in detail.

TABLE IX.

Thyroid.

	Mice with Spontaneous Tumor.	Old Controls.		Young Controls.
		♀	♂	♀
Number examined	87	25	41	46
Normal	10 (11%)	9 (36%)	31 (75%)	38 (84%)
Slight acinar hypertrophy....	4 (4%)	0	0	4 (8%)
Exophthalmic type goiter....	24 (27%)	0	0	4 (8%)
Colloid goiter	26 (29%)	6 (24%)	0	
Papillary cystadenoma.....	23 (26%)	10 (40%)	0	
Fibrosis	6 (6%)	0	10 (25%)	

Adrenal. — The adrenal gland, held to be chiefly responsible for cancer formation by Sajous, had as its most frequent change fibrosis of varying degree. This fibrosis, which is not restricted to the tumor-bearing group, is mainly confined to the cortex of the gland. Associated with the lesion there is a varying amount of degeneration of the cells, chiefly of a fatty type. Accessory adrenal glands situated within the gland capsule, usually in the cortex of the main gland, were rather frequently encountered in the tumor-bearing group. Fibroid changes of a similar type were not observed in the adrenal gland of rats. The appended Table X. gives the more exact details.

TABLE X.

Adrenal.

	Mice with Spontaneous Tumor.	Old Controls.		Young Controls.
		♀	♂	♀
Number examined	90	22	45	50
Normal	14 (14%)	6 (27%)	39 (86%)	45 (92%)
Accessory gland.....	8 (8%)	0	3 (6%)	1 (2%)
Fibrosis	74 (82%)	16 (72%)	6 (13%)	4 (8%)
Fatty degeneration.....	12 (13%)	2 (9%)	0	0
Round cell infiltration.....	3 (3%)	0	0	0

To summarize, the chief changes noted are of a type more or less constantly associated with increasing age. The only changes apparently characteristic for the tumor-bearing group are the thyroid lesions. But these changes are not restricted to tumor-bearing animals and are, therefore, not specific. The study of the thyroids in an additional control group of twenty old non-tumor-bearing males of the same strain as the tumor-bearing females revealed thyroid lesions similar to the tumor-bearers.

Our experience shows, therefore, that mice like dogs, trout, and some of the herbivora from certain districts may show marked thyroid changes due to causes as yet unknown, but not in the least correlated with the presence of neoplasms. If males from the same locality and strain as the tumor-bearers had not been examined faulty results would have been obtained, thus again emphasizing the need for extreme caution in drawing conclusions and for the use of every possible control, particularly in cancer research.

DESCRIPTION OF PLATE X.

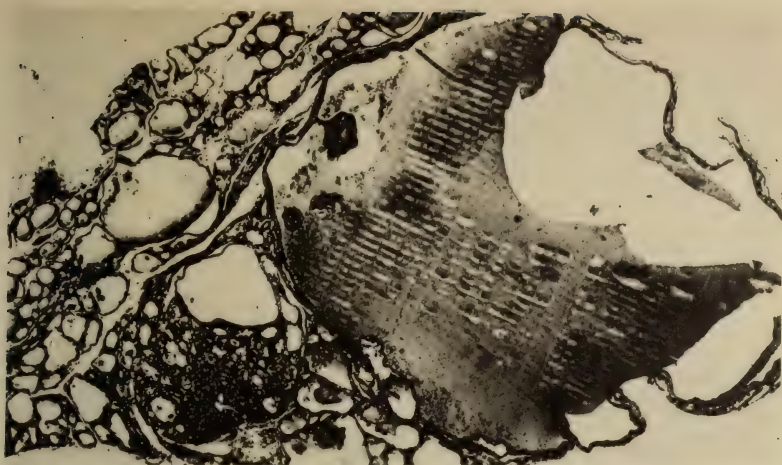
FIG. 1. — Thyroid showing slight papillary cystadenoma.

FIG. 2. — Thyroid showing fibrosis associated with colloid goiter.

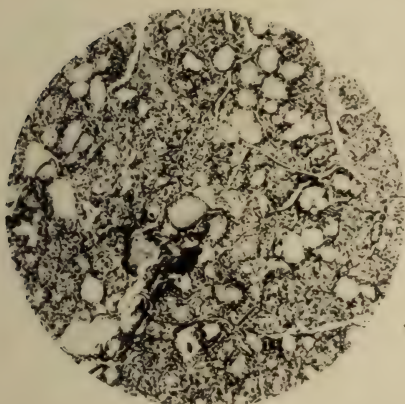
FIG. 3. — Thyroid of exophthalmic type.

FIG. 4. — Papillary cystadenoma.

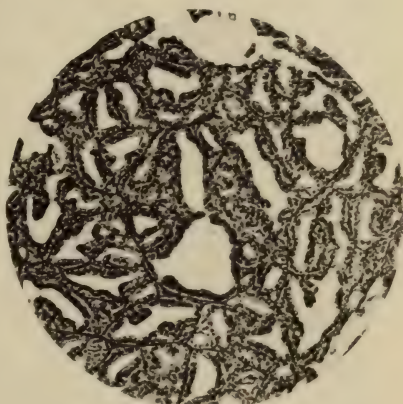
FIG. 5. — Colloid goiter.



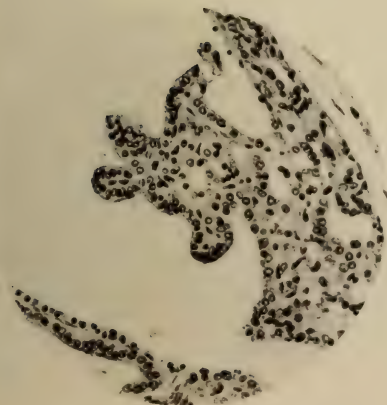
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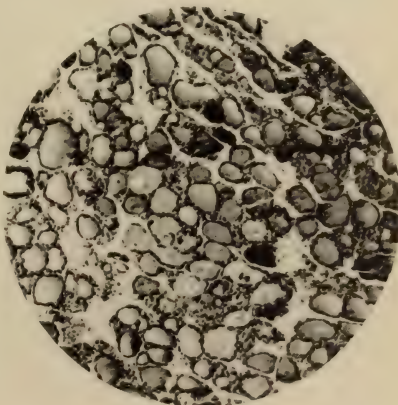
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VASCULAR CHANGES FOLLOWING INTRAVENOUS INJECTIONS OF FAT AND CHOLESTERIN.*

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Much attention has recently been given to changes occurring in various tissues from the feeding of certain abnormal diets. These experiments have been mainly confined to rabbits, which, by forced feeding, were given animal proteins, fat, lipoids, and cholesterin. The work was initiated by the Russian school in St. Petersburg. Ignatowski was the first to observe tissue lesions resulting from abnormal foods. He was led to believe that the injury was induced by the animal proteins present in the diet. Subsequently, however, the work was given a more critical analysis and it was shown by Stuckey that the protein diets (egg white and meat juice) had very little effect in inducing tissue changes, but that substances containing fats, lipoids, and cholesterin (egg yolk, brain, liver substance, and milk) were active in producing tissue changes. In segregating the various constituents of these diets Stuckey found that the neutral fats played very little part, while Wesselkin found that pure lethicin was also without effect. There remained, therefore, one important ingredient common to these foreign diets, cholesterin, which might be suggested as the active agent. This was the more obvious from the work of Chalatow, who observed that the tissue changes induced by these dietary experiments were associated with isotropic and anisotropic deposits in the liver, substances which had been shown by Adami and Aschoff to contain cholesterin. The proof of this surmise was made by Anitschkow, who fed animals with various amounts of pure cholesterin.

The experiments have been repeated by many and, of late, numerous reports are available on the importance of the cholesterin content in the diet and the influence of this

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upon the tissues. It has been shown by Rothschild and Sternberg that an increase of the cholesterin in the diet leads to a hypercholesterinemia as well as an abnormal deposit of cholesterin compounds in the form of esters in the adrenal, liver, spleen, ovary, and the arteries. During the increased accumulation of these cholesterin compounds in the organs there is also an architectural change which has formed the basis of a number of interesting studies. These tissue changes have in the main to do with the increased activity of particular cells. These cells, endothelial in nature, show the ability to store the cholesterin esters of the blood and appear to relieve the load imposed upon the body fluids. The adrenal is unusually active and appears to act as an intermediate organ for the final disposal of the cholesterin esters. Apparently, however, these cells of the adrenal cortex are inadequate to care for the cholesterinemia, so that cells of similar nature and function appear in unusual situations to undertake the work. The ovary may be found to consist of one immense corpus luteum-like structure; the spleen shows an unusual endothelial proliferation with the loading of these cells by anisotropic masses; the liver cells likewise partake in an active handling of cholesterin esters with a not uncommon precipitation of cholesterin spicules within their substance; and finally the arteries show an abnormal response with the development of plaques upon the intima consisting of endothelial cells, loaded with cholesterin esters and giving a microscopical appearance to the cell clusters, like that of the adrenal cortex. It thus appears that the hypercholesterinemia induced by the feeding of cholesterin calls forth a curious type of endothelial proliferation which attempts to deal with the abnormal products by a latent function.

The presence of cholesterin crystals in tissues has long been observed. The conditions under which this cholesterin appeared have usually been those of degeneration, and more particularly degeneration of a chronic type. We have had, however, little evidence of the source of this cholesterin in that the amount which appeared in certain areas was quite

out of proportion of the normal content of these tissues. In the arteries, for example, cholesterol crystals are commonly noted in the atheromatous areas, while the amount of cholesterol in any of the tissue cells of the arteries is almost a negligible quantity. Furthermore, cholesterol masses may be observed in the necrotic areas of tumors and, as well, scattered through areas of chronic inflammation of the breast, ovary, tubes, and epithelial structures. In all of these instances the cholesterol was observed in the nature of its irregular crystals and not uncommonly the crystals formed the center of giant cell proliferation. The presence of giant cells about cholesterol crystals has been so frequently observed that some have come to speak of them as cholesterol giant cells. With it all, however, little has been done to indicate the manner in which the cholesterol was deposited in these situations. It is obvious that the pure substance was not transported as such to these localized areas and equally obvious that the cholesterol did not rise in these tissues by synthesis. Corper has shown that cholesterol in the form of its crystals is an extremely inert substance. In his experiments by placing chemically pure cholesterol into the animal tissues he found that after a year only small amounts disappeared and that almost the entire unaltered original crystals could be recovered after two years of time. It is very evident, therefore, that the metabolism of cholesterol is carried on mainly when it is in association with other substances either holding it in solution or as compounds.

The necessity of cholesterol being contained within a vehicle is illustrated in the feeding experiments. The best results are obtained when the relatively inert cholesterol is offered in an available condition. This has been accomplished by dissolving it in various oils (sunflower oil, olive oil) or when the cholesterol is combined with fatty acid compounds, as will be illustrated in our subsequent report. The use of this cholesterol soap mixture or compound has been shown by McMeans of our laboratory to be in a very available form for use by the tissues. When, however, these

mixtures are fed to animals it is not clear in what manner they pass through the intestinal wall. It has been suggested that true cholesterin esters are observed as such and that these cholesterin esters are to be demonstrated in the fluid of the thoracic duct (Landau). We have, however, in our feeding experiments not been able to demonstrate anisotropic bodies in the milky lactiles of the treated animals. It may be that the cholesterin esters under these conditions do not show their anisotropism.

When the pure crystalline cholesterin is added to the animal diet it is found that a much smaller quantity reaches the animal tissues (Knack). It would appear that some of this inert cholesterin is taken up, probably by passing into solution in some of the oily substances present in the ordinary diet of animals. Knack, however, was unable to obtain tissue changes comparable with those of others, even though his experiments were prolonged over a longer period of time and a much larger quantity of cholesterin was given.

It appeared to us sufficiently interesting to observe whether the artificial intravenous introduction of cholesterin in different vehicles brought about some of the changes in the tissues comparable to those of the feeding experiments.

A series of experiments was undertaken to determine the direct effect of various combinations of cholesterin, fats, and soaps upon different tissues and particularly upon the arteries. In the first group, olive oil and cotton seed oil were used as solvents for cholesterin, while individually each was also tested out for its irritating or stimulating qualities to the tissues of rabbits. A solution of 1 in 15 of cholesterin in these oils was found to be close to the point of saturation and served as a standard solution. It was found that at room temperature this concentration of cholesterin in olive oil gave a semi-solid material, but when warmed to body temperature a clear solution was obtained. These cholesterin oils were inoculated intravenously twice or three times a week into young rabbits and the results observed at intervals from six days to three months. The cotton oil

mixture was soon discarded, as there was evident toxic effect with rapid emaciation — presumably from constituents of the cotton oil. On the other hand, the cholesterin olive oil mixture was inoculated intravenously over long periods of time without serious untoward effects, in fact the animals gained weight. Quantities varying from .5 to two cubic centimeters were given, the usual dose being one cubic centimeter. In no instance did the animal show signs of pulmonary embolism although, as it was subsequently demonstrated, great numbers of the capillaries of the lung were completely occluded by the oily mass.

In a second series the cholesterin was combined with a pure sodium oleate solution in a definite proportion. Merck's sodium oleate was used. A five per cent solution of sodium oleate was combined with five grams of cholesterin. In this proportion it was found that all the cholesterin was taken up, so that no free crystals remained, and the fluid was changed from the transparent yellow solution of sodium oleate to a milky permanent emulsion in which great numbers of anisotropic globules were seen. This emulsion was prepared by heating in a water bath and was found to retain its physical properties for many weeks. It was interesting, too, to observe that the previous intense hemolytic qualities exhibited by the pure sodium oleate solution had almost entirely disappeared. We found, however, that this hemolytic quality of the sodium oleate cholesterin emulsion could be entirely masked by adding an equal quantity of human serum, or by increasing the cholesterin concentration to 7.5 per cent.

In this final combination the anisotropic bodies were as marked as in the original emulsion. It was furthermore observed that this fluid had some antiseptic but not disinfectant properties. Quantities varying from one to two cubic centimeters of this fluid were inoculated intravenously into rabbits without apparent injury. The animals received treatment two or three times a week over a period up to three months.

SERIES I.

Cholesterin olive oil.

Rabbit.	No. of Injections.	Total Amount Injected.	Length of Treatment.	
1	1	1 cc.	4 days.	Killed.
2	4	3.5 "	7 "	"
3	7	7 "	22 "	"
4	7	7.5 "	25 "	"
5	11	11 "	34 "	"
6	12	15 "	45 "	"
7	13	13 "	43 "	"
8	13	15 "	64 "	"

In the first series almost the entire oil cholesterin mixture was filtered out by the capillaries of the lung. Some, nevertheless, escaped, and were found in the renal glomeruli and the small vessels of the heart, liver, and spleen. Relatively, however, the quantity of demonstrable oil mixture in tissues other than the lung was very small. Even after a few doses the lung appeared suffused with oil droplets, each of which retained its position within the capillary channel. The lung tissue was firmer and did not collapse as readily as the normal tissue. The vessels and capillaries which were still patent were dilated and engorged. In the areas of capillary occlusion the alveoli appeared shrunken, in part as if by collapse, but also due to a reactionary thickening of the alveolar walls. Thus, all through the lung were scattered many little nodules, so that the section to the naked eye somewhat resembled a miliary tuberculosis.

During the first few weeks the fat alone was demonstrable as oil globules lying free within the capillaries or in vacuoles of large phagocytic cells. Later, however, the cholesterin crystals made their appearance apart from the oil globules and became embedded in large foreign body phagocytes. Gradually these crystals were separated from the region of

the demonstrable oil globules, with the development of a marked proliferative lesion surrounding them. It is more than probable that these cholesterol crystals were separated from the oil droplets through intracellular lipolysis. Hence, in following the fate of the ingested oil mass, attention must be given to the fat particle on the one hand and the cholesterol portion on the other.

Both olive oil and cotton seed oil when alone inoculated into the ear vein are rapidly removed from the blood stream by the lung. Clogging of the fine capillaries and arterioles was found in all portions of the lung tissue and in most instances was complete. However, not a few vessels remained patent. Within a few days a marked reaction was seen in the vicinity of the embolic fat in which the tissues of the vessel wall took a prominent part. The growth of the lining endothelial cells penetrated the oil globules or completely surrounded the mass. Where proliferation was active the globules appeared to split into smaller portions which became phagocyted by different endothelial cells or in different portions of the same cell. Lymphocytes appeared in small numbers in the reaction, but polymorphonuclear leucocytes were wanting. It was very remarkable to observe the size to which the endothelial cells grew when phagocytizing these large oil masses. The protoplasm was stretched so that only a narrow rim bounded the periphery and the nucleus was displaced and often flattened. With the removal of the fat from such sections these endothelial cells had the appearance of ordinary fat cells. It was not uncommon to find several giant cells occupying the lumen of the arterioles and containing fat globules. On the other hand, proliferative changes in the lining of the larger vessels led to an irregular thickening along one border of the lumen. Such reactions of irregular vascular thickening simulated the picture of an early endarteritis, or better of thromboangitis obliterans.

With the proliferative reaction occurring in the alveolar walls the fat globules came to occupy positions which could no longer be associated with the vascular structures. Fat

globules were scattered through the thickened tissue and were contained within large plasmodial cells, some of which had vacuoles. These interstitial cells had a single well staining and centrally placed nucleus. Within these cells the fat gradually changed its globular appearance to that of a granular one with less affinity for the fat stains. With Nile blue staining, these granules were blue or purple.

These proliferative changes could be observed well advanced at the end of the first week and the reaction in as far as the proliferative response of the capillary endothelium was similar whether the fat was introduced alone or in combination with cholesterin. No evidence of free cholesterin was observed in these tissues until toward the end of the second week. From this time on cholesterin crystals began making their appearance in the tissues, probably in relation to the destruction and absorption of the fat.

Interesting as is the tissue reaction in the alveolar walls of the air sacs to the presence of these foreign materials, we centered our attention, in the main, upon the changes developing in the arterial walls. As indicated above, the foreign material when introduced in the nature of oil mixtures was for the most part removed by the lung capillaries, but nevertheless a certain quantity did pass through these minute channels to escape into the systemic arterial tree. In part, these fatty bodies are removed from the blood by the small capillaries in all parts of the body, the quantity held in any particular tissue being dependent upon the size and tortuous nature of the vessels. No fatty substances were demonstrated in the large arteries, nor was there any evidence that the lining wall of these attracted by phagocytosis the fatty substance into their tissues. Throughout the experiments the large arteries were without change. The aorta was entirely free from any form of sclerosis and there was no evidence of a process of cholesterin-ester phagocytosis as described by Anitschkow. This was true both for the experiments where cholesterin was embodied in solution in oil as well as where the cholesterin-ester was introduced as an emulsion.

On the other hand, wherever the introduced fatty materials were in direct contact with the vessel wall, either through a process of embolism or where small droplets remained adherent in the small arterioles, a reactionary response could be followed in the vessel. Obviously, the frequency of such vascular changes was in direct proportion to the amount of the cholesterolin mixture present as demonstrable masses in the organs. The lung tissue showed the greater frequency of arterial change and next to this the kidney, heart, and spleen. This is interesting in view of the fact that reactions in the pulmonary arteries are much less frequent under other conditions than those in the systemic channels.

The reactions observed in the arteries were of two kinds. On the one hand, an active proliferation on the part of the endothelial elements of the capillaries and smallest arterioles gives rise to a new tissue mass which tends to occlude the vessel and to remove the foreign fat by phagocytosis. On the other hand, the reaction in the larger arterioles shows not only a proliferation of the endothelial elements but deeper changes in the vessel wall, materially altering its architecture.

In our present study the first of these responses, in which only the smallest vessels took a part and in which the endothelial cells alone were stimulated to growth, has no immediate interest in our problem. In the second instance, however, we have a greater interest. Here we find an endothelial proliferation altering the size of the lumen. The caliber of the vessel is materially diminished. In the new growth of the lining membrane of the arteries many layers of cells are produced and all of them are active in dealing with the foreign fatty materials adherent to the wall. Globules and granules of the fat mixtures are contained in the protoplasm. Some of them have the character of the original material, others have doubly refractile qualities not previously possessed by the oil mixture and some show the character of an unsaturated fatty acid. Accompanying this endothelial proliferation there was also observed some increase of the underlying connective tissues. This deeper proliferation furthermore assisted in distorting the arterial

lumen. With this the almost uniform finding of splitting of the internal elastic lamina was most interesting. This elastic membrane was found broken into multiple layers and the extent of its change was in proportion to the response in the overlying tissues. The elastic fibers were not only split into many strands but alteration in their chemical composition could be demonstrated by staining reactions. At times, small quantities of fat were present in the fibers, while others had advanced to calcification. No change was observed in the media, save when by the amount of tissue reaction the intima encroached upon the muscular wall, compressing and thinning it.

With the splitting of the internal elastic lamina, tissue elements made their appearance between the fibers. These cells commonly contained fatty materials, some looking like connective tissue cells, others simulating the large phagocytic endothelial cells of the surface. A proliferation of deep intimal muscle cells taking part in the reaction could not be determined. Thus a type of intimal sclerosis, simulating atheroma, was obtained. In the advanced lesions the appearance was not unlike that of nodular endarteritis of human arteries in which a deep fatty deposit is covered by a cap of subendothelial sclerosis.

From these experiments it would appear that one or both of the elements in our cholesterol oil mixture was a factor in producing intimal sclerosis. Olive oil when used alone gave no evidence of arterial lesions in the lung save the endothelial proliferation of the capillaries. Repeated intravenous inoculations of olive oil showed only a slight deposition of fat droplets in the alveolar walls. Some large phagocytic cells made their appearance, but never in the numbers as previously observed. Moreover, the fat content of the lung disappeared much more rapidly than in the presence of cholesterol. It appeared to us that olive oil alone was less irritating to the vessel walls, and that the peripheral digestion of the oil droplets by serum enzymes was sufficient to reduce their size and liberate them into the distal circulation. The intense phagocytic activity by endothelial cells of the cholesterol oil prevented such a fate for the

material. It would appear, therefore, that the greater deposition of the cholesterin mixture in the lung resulted from the irritative qualities of the cholesterin component.

SERIES II.

Sodium oleate cholesterin.

Rabbit.	No. of Injections.	Total Cholesterin Injected.	Length of Treatment.
1	1	150 mg.	1 day. Killed.
2	7	210 "	30 days. "
3	8	240 "	32 " "
4	3	450 "	14 " "
5	5	500 "	10 " "
6	14	2150 "	51 " "
7	15	1750 "	23 " "

The results in the second series were not accompanied by as marked results as the first. They were, however, more widely distributed and not confined to the pulmonary system as when using the cholesterin oil. We had hoped that by the introduction of a cholesterin combination, in a more readily assimilable form, tissue reactions would result more easily. It was soon found, however, that two factors are essential, first, an adequate time of treatment and the introduction of the cholesterin mixture in sufficient quantity to produce a definite hypercholesterinemia. Hence, the results of the first experiments in which a relatively short period of time and small quantity of the mixture was used were negative. No evidence was obtained of abnormal tissue reaction or of the deposition of the introduced materials. Where larger quantities of the mixtures were given, slight reaction was obtained in the spleen and liver, while in one case a slight superficial fatty change was observed in the aorta.

Some difficulty was experienced in the use of the cholesterin oleate mixture. It was found that a considerable inflammatory reaction occurred at the points of intravenous

injection and this was then followed by an occlusion of the channel. Thus it was not possible to continue the experiments on the animal in the absence of suitable veins for inoculation. The irritating character of the fluid was further demonstrated by the intraperitoneal inoculation of guinea-pigs. Within eighteen hours much of the inoculated material was precipitated about the omentum and there was evidence of reddening about the peritoneal tissues. Anisotropic bodies could be demonstrated in the folds of the omentum as well as in the leucocytes and endothelial cells of the exudate. After several days the omentum appeared fatty and fine acicular crystals were scattered through its tissues. The large phagocytic cells were found to contain granular fat and cholesterin, while the fibrous tissue cells were found proliferating and loaded with fat masses. At this time it was found that the material both within and outside of cells had lost its anisotropic quality, and the cholesterin was deposited in crystalline form.

The cholesterin sodium oleate mixture which had been given intravenously showed no predilection for the lung tissues. Its emulsion was sufficiently fine to permit the globules to circulate freely. After a single or several injections no trace could be found of the inoculated material. It would appear that the mechanism for handling this cholesterin combination could easily take care of the amounts introduced. It was impossible to say that a greater quantity was present in those tissues — adrenal — which are believed to take care of cholesterin metabolism.

In the subsequent and more prolonged experiments where larger amounts were given we did believe that the adrenals were enlarged, and in these a proliferative response of the endothelial cells of the spleen was observed. These spleen cells contained fatty substances and occasionally crystalline spicules. In only one instance did we observe the accumulation of fatty globules, some of which were anisotropic, as well as some acicular crystals in the liver. The fatty streaks in the aorta which were also encountered in this case showed the presence of small aggregations of endothelial cells loaded

with anisotropic fat, as has been described in the cases of experimental dietary cholesterinemia.

The analysis of the results we have obtained by the intravenous introduction of cholesterin mixtures indicates two distinct processes of dealing with the materials we have used. On the one hand, while the presence of oily globules in themselves stimulate endothelial proliferation and phagocytosis of these globules, the presence of cholesterin in the oil appears to act as a greater stimulus to cell activity. We have previously demonstrated that the phagocytizing endothelial cells are capable of digesting fats within their protoplasm. When cholesterin oils are phagocytized the cells appear to separate the oil and cholesterin, dealing with each individually. The oil is decomposed by intracellular lipolysis, while the cholesterin remains as an inert material within the cell subsequently giving rise to giant cells with clefts. These cholesterin giant cells may be readily reproduced in the lung tissue, and give rise to small nodular tissue masses which remain for long periods of time. The vascular changes develop opposite the points where the lining endothelial cells have been stimulated by the deposit of the cholesterin oil. Nodular proliferations of the lining membrane lead to distortion of the lumen. The muscle fibers of the neighboring media also take up some fat. Processes of calcification have been encountered in these fatty deposits. On the other hand, the use of cholesterin in a fine emulsion of cholesterin oleate gave rise to a more systemic reaction not dependent upon localized phagocytic activity. Here it would appear that the tissues normally dealing with blood cholesterin responded and it was in these tissues that cellular changes resulting from excessive cholesterin in the blood took place. These latter results, observed in the adrenal, spleen, and liver as well as the aorta, simulate to a mild extent the changes observed in hypercholesterinemia of the feeding experiments.

CONCLUSIONS. — Fat introduced intravenously is mainly filtered out by the capillaries of the lung where it is in part digested by the serum lipase. The presence of fat emboli

stimulates proliferation of the lining endothelial cells, which are phagocytic for the fat. An intracellular lipolysis is carried on by these endothelial cells.

The phagocytic activity of the vascular endothelium for fat is enhanced by the presence of cholesterol. The phagocytosis of the cholesterol fat leads to a dissociation of these bodies, leaving the cholesterol as relatively inert bodies within the endothelial cells. During the progress of the phagocytosis of the cholesterol fat, the associated proliferative response of the endothelium may lead to a great distortion of the vessel lumen.

The introduction of cholesterol emulsions into the systemic circulation stimulates a response in the endothelial tissues of various organs (spleen, adrenal, arteries) as well as an accumulation of anisotropic cholesterol compounds in them and the liver.

The part played by the arteries in the presence of abnormal quantities of cholesterol in the blood is through the activity of the endothelium of the intima. This tissue has phagocytic properties for cholesterol in solution in fats, as well as a functional activity in dealing with cholesterol compounds.

The subsequent development of calcification in these areas has a direct relation to this local fat metabolism.

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SPONTANEOUS PRIMARY TUMORS OF THE LIVER IN MICE.*

STUDIES ON THE INCIDENCE AND INHERITABILITY OF SPONTANEOUS TUMORS IN MICE.

(*Sixth Communication.*)

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The distribution of various types of tumors in different species of animals show variations that are often difficult of interpretation. Notable among such peculiarities in distribution are cancer in the stomach and uterus, which are most common in man and extremely rare in every other species; or, in mice, the great frequency of mammary gland carcinoma and papillary tumors of the lungs, which together account for at least eighty per cent of all the spontaneous tumors of mice. In respect to tumors of the liver, however, the incidence seems to correspond more closely in mice and men, for the entire literature on primary spontaneous tumors in mice contains, as far as we can find, but one report of a primary epithelial tumor of the liver, this having been described by Murray.¹ Tyzzer² has reported a suspected primary sarcoma of the liver. Apparently, then, such liver tumors are extremely uncommon in mice, as they are in man. Winternitz,³ in a review of the literature on primary carcinoma of the liver in man, finds various statistics giving an incidence of .028 to .3 per cent of all autopsies. In three thousand seven hundred autopsies at Johns Hopkins Hospital there were three positively determined primary carcinomas of the liver, or .08 per cent. One of us has observed four such cases in about one thousand five hundred autopsies, or nearly the maximum figure mentioned in the statistics quoted. Primary sarcomas of the human liver are so rare that they would affect the figures but little. Adenomatous

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growths are, however, more common, but we have found no statistics concerning them; and, furthermore, the difficulty of differentiating between neoplastic and inflammatory or compensatory hyperplasias of liver tissue would render such figures of little value.

In other animal species the proportion of liver tumors varies, but seems to be usually higher than in man. Casper⁴ makes the statement that true primary sarcomas of the liver, composed of either round or spindle cells, are frequently seen in horses, bovines, dogs, and goats. Adenomas are also described as frequent, reproducing either liver cells or bile duct cells, as in man. Of fifty-one carcinomas in dogs that he collected, seven were primary in the liver. He says that in the liver of old dogs, and also in horses, primary carcinoma is frequently seen. According to his descriptions these tumors in animals resemble closely those seen in man, for they may consist of either liver or duct cells, appear as solitary massive growths, multiple nodular tumors, or diffuse infiltrating neoplasms, all with a tendency to break into the large portal or hepatic veins. Furthermore, he says "the separation of adenomas from carcinomas is often difficult, and in such cases one must be content to designate them as 'Adeno-carcinoma,' " a statement equally applicable to human liver carcinomas.

Williams,⁵ in recapitulating the literature on tumors in animals, cites the following statements concerning cancer of the liver: Of one thousand three hundred and twelve cases of cancer of common domestic animals, only forty-two were in the liver (no data as to how many of these were primary). Of seven hundred and thirty-eight cases of canine cancer, Sticker found three per cent in the liver. In the cat cancer occurs in the liver. In cows cancer of the liver and stomach heads the list of most pathologists, but Sticker gives the uterus as the most usual site. In pigs the kidney, skin, and liver are the commonest seats of initial manifestations, but all tumors are rare in pigs. In sheep, of the few recorded cases, in the great majority the growth arose in the liver.⁶ However, if one looks over the protocols given

in Sticker's paper,⁷ it seems probable that many of the cases recorded as primary are really secondary. Of the tumors of the liver in dogs, for example, only three seem surely primary from his description.

In the Pathological Museum of the University of Chicago we have a typical primary carcinoma of the liver, of the liver-cell type, found in a dog used for experimental work. The miscellaneous lot of animal tumors described by Murray as sent to the London Laboratories also includes one columnar cell carcinoma in the liver of an old female dog, and a "malignant adenoma" of the liver of the cow.

In most wild animals also the liver seems seldom to be the point of origin of cancers. Thus in the two thousand five hundred and thirty-three wild animals autopsied by Fox,⁸ there were thirty-four tumors, which included one liver tumor only, this being an adenoma of the liver of a woodchuck (*Arctomys monax*). Wild rats would seem to be an exception, according to the observations of McCoy,⁹ who found in one hundred thousand killed in plague prophylaxis, one hundred and three with tumors, among which sarcomas of the liver and subcutaneous (mammary) adenomas were most frequent. Of the one hundred and three tumors no less than twenty-two arose in the liver, eighteen being sarcomas, two fibromas, one fibro-adenoma and one angioma. These liver sarcomas commonly produce metastases in the omentum, but, contrary to the usual observation in similar tumors in man, not at all in the lungs. This observation is supported by Woolley and Wherry,¹⁰ who describe twenty-two tumors in wild rats including three sarcomas of the liver. In both reports the common association of these neoplasms with cestodes is emphasized. McCoy¹¹ more recently reported on the occurrence of tumors in ground squirrels killed in the California plague work. Of two hundred and fifty thousand such animals, only eight showed tumors, of which two were adenomas of the liver, both apparently of benign character, and in one the blood spaces were conspicuous as in an angioma. No mention is made of cestodes or other parasites in connection with these tumors.

In view of the proportion of sarcomas in the liver in rats, the absence of reports of such growths in mice is indeed striking. The California writers called attention to the coincident occurrence of hepatic sarcoma and cestodes, but in the Slys stock cestode infection of the liver has been common, with no cases of hepatic sarcoma in such animals.

That in all the literature on mouse tumors we can find but two cases of a tumor of any kind in the liver is also remarkable, in view of the number found with this stock. Haaland's report¹² on three hundred and fifty spontaneous tumors arising in three hundred mice in the London Cancer Institute includes no description of any growth whatever in the liver. However, in his series but fifty-five tumors had arisen outside the mammary gland, including the lung adenomas, a fact that suggests that the tumors in this series had been detected more frequently by external examination than by systematic autopsies. The same statement probably holds for Jobling's series¹³ of forty-one tumors in twenty-six mice, which series also includes no liver tumors. Murray's case of adenoma of the liver in a mouse was observed in an old female mouse that had died after extirpation of a primary sarcoma of the mammary gland. He described it as follows¹:

"A mouse with a large mammary carcinoma of the right inguinal region died a few hours after extirpation of the tumor. At the autopsy a mass of growth was found in the right lobe of the liver. The growth measured one centimeter in its greatest diameter, was slightly flattened and formed a lenticular swelling near the sharp margin of the organ with only a narrow thin edge of liver substance at its free border. On section the growth was white in color with punctiform hemorrhages, the cross-sections of blood vessels. The ordinary brownish coloration of liver was absent, and the oval section of the growth was sharply demarcated from the thin peripheral wedge of liver tissue and from the main mass of the organ. On microscopical examination the white tumor-like mass showed a very close resemblance to normal liver structure. The arrangement of the cells is irregular, however, here and there are indications of a radial arrangement as if around a central vein, but portal tracts with bile ducts are absent. The cells are highly atypical, of very varying size. The reticular fibrils of the protoplasm are much finer than in normal liver cells, and are frequently arranged in parallel bundles which stain deeply

with the methylene blue of Giemsa's solution and with iron-hematoxylin, in this respect resembling the protoplasmic fibrils of the cells of the pancreas. The nuclei present the most bizarre variations. Some are small and dense, others are large and vesicular with enormous nucleoli. In many cells two or more nuclei are present, but mitoses are extremely scanty and evidently highly pathological with clumping of the chromatin. Where the growth adjoins the surrounding liver substance the cells of the latter are compressed and atrophied."

No metastases were found, and transplantations into sixty normal mice produced no growths. His diagnosis is "adenoma, probably malignant." In all respects this tumor corresponds with the adenomas we have found in mouse livers.

Tyzzar² described his case of sarcoma of the liver as follows:

"Throughout the liver are small whitish nodules, many of which are sharply circumscribed and rounded. None exceeds three millimeters in diameter. The spleen is very large, its surface puckered in places and presents several ill-defined whitish areas. The nodules in the liver are found to be composed of cells of the nature of connective tissue cells, associated with collagen fibrils. These cells vary in size and shape, and are often elongated or irregular. Some cells are multinucleated. Others present a centrosome situated opposite an indentation of the nucleus. Scattered here and there throughout the tumor nodules are isolated degenerating liver cells and small bile ducts. The tumor has grown diffusely throughout the liver so that the liver columns are compressed. The spleen also presents several masses of atypical tissue such as that present in the liver. Few mitotic figures are present."

The statement that the spleen also presents masses of the same tissue, and that the nodules in the liver are small and multiple ("there is no nodule of such size that it can be considered the primary growth"), raises the question of the primary character of the growth in the liver. If primary in the liver the metastases in the spleen against the current are difficult of explanation; furthermore, in mice as in men the spleen seems to be an unusual site of secondary growths. However, if the growths in the spleen are primary, the condition in the liver is easily understood.

Occurrence of primary liver tumors. — In ten thousand autopsies performed on mice of all ages from the Slye stock, which have died natural deaths without experimental inoculation or other manipulation, there have been found twenty-eight growths in the liver, of such a gross and microscopic character as to warrant a diagnosis of adenoma, an incidence of 2.8 per thousand. The only influence that might modify the rate of occurrence is the heredity of the animals, since they have all been bred with reference to the incidence of cancer. There are, in these ten thousand mice, strains that are notably cancerous, and strains that are free from cancer, the influence of which facts has been considered elsewhere.

A certain proportion of the ten thousand mice had not reached an age of one year, before which tumors rarely occur, so that the incidence in mice of cancer age is considerably higher than three per thousand.

As to sex, there were fourteen females and fourteen males, this being in contrast to the sex relation in most series of mouse tumors previously described, but more nearly reached in our primary lung tumors, in which there were 42.6 per cent in males and 57.4 per cent in females. This indicates that the predominance of female tumor mice is simply due to the predominance of mammary gland tumors.

Whereas in man cirrhosis of the liver seems to play such an important part in predisposing to cancer, mice are entirely free from this condition. In these ten thousand autopsies we have never seen a typical cirrhosis. Only one mouse showed a condition even resembling cirrhosis, but in this liver, which was rough and nodular on the external surface, the change microscopically was that of a liver in which an extensive peripheral necrosis had occurred with reduction in size of the lobules and more or less pigment and débris remaining about them, but with very little growth of connective tissue. The liver cells remaining were enlarged and the general appearance is that of recovery from an acute destructive lesion.

Mice very commonly exhibit inflammatory processes in

the liver, and cestode infection of the liver is frequent, but these processes do not result in the production of anything resembling an atrophic cirrhosis, such as is so commonly seen in other laboratory animals, especially the rabbit. In none of the liver tumors of this series has there been any evidence of any particular inflammatory or other pathological condition acting as the predisposing cause of the tumor. In but three were cestodes found, in spite of the frequency of this infection in the stock at times, and in no case did the tumor growth show any connection with or relation to the cestode lesion.

Of the twenty-eight adenomas there are three that are certainly malignant, according to their structural characters, one of these having produced multiple metastasis in the lung (Figures 1, 2, 3). There are three others that show histological features strongly suggestive of malignancy, and the remaining twenty-two are all certainly or probably benign. We thus have a graded series ranging from unquestionable liver cell carcinoma with metastases, through local but malignant adeno-carcinomas to the simplest of benign liver cell adenomas. There are also growths in which it is difficult to determine whether they are simple localized inflammatory or compensatory hyperplasias of liver cells or true adenomas, none of which has been included in this series. We have, therefore, quite the same conditions as regards the tumors of the liver cells in mice as are familiar in the liver cell adenomas of man, with approximately the same proportion of malignancy.

We have never found in the mice a tumor that showed a bile duct structure, although in man these constitute a considerable proportion of the tumors of the liver. Jaundice or ascites did not occur in any of these cases. There were no tumors found in the gall bladder or bile ducts.

Anatomical characteristics.—The gross appearance of these tumors is rather uniform (see Figures 1 and 4). They exhibit a rounded growth, covered by the original capsule

and not adherent to surrounding viscera, usually pedunculated if of any considerable size, but the smaller tumors appear simply as nodules in the liver substance. Commonly they are lighter colored than the normal liver, but sometimes they are mottled or uniformly darker, and often distended vessels are conspicuous on the surface. They vary in size from two or three millimeters up to great growths of twenty to thirty millimeters diameter, larger than the original liver and distending the abdomen.

In these liver tumors we see the same tendency to multiple formation of primary tumors which is so noteworthy a feature of mouse cancer, especially in strains bred for tumor ancestry. Of the twenty-eight cases under discussion, ten showed more than one tumor, as follows:

Three with multiple liver tumors, in each case one being malignant and one benign (a similar coincidence of benign and malignant neoplastic structures has also been noted in human liver cancer).

Two with lung adenomas of benign type.

Two with malignant papillary adenoma of the lung.

One with sarcoma of the mammary gland.

One with a benign adenoma of the mammary gland.

One with a carcinoma of the mammary gland.

It is noteworthy that there was but one case of mammary gland cancer among this series, although these constitute fully or more than half of all primary tumors in mice.

Microscopically the typical adenoma of the mouse liver consists essentially of a circumscribed area or nodule of liver cells, differing from normal tissue by the variability in size and staining reactions of the cells, the marked irregularity in the arrangement of the cords of liver cells, the absence of regular relation to central veins, the lack of lobule formation, and especially in the absence of bile ducts or other evidences of the portal systems in the adenomas. They are never encapsulated by a layer of connective tissue, but are often marked off by a surrounding zone of compressed liver cells (see Figure 5). The expansive character of growth is usually marked. Occasionally the large irregular cords of

adenoma cells seem to be continuous with apparently normal cords at the periphery of the growth, a condition which has been repeatedly observed and commented on in human liver cell tumors. A conspicuous feature in most of the growths is the formation of extremely large liver cells, sometimes multinuclear, but oftener with a single giant nucleus. The sinusoids present the same relation to the liver cells as in the normal liver, although often they are much wider and highly irregular in size and shape, forming large blood filled spaces. Mitotic figures are occasionally but infrequently found in the benign growths; amitotic division was not often noticed as in the lung tumors. Retrogressive processes are common, especially areas of necrosis; fatty infiltration is not so often seen, but the cells are often vacuolated to a marked degree. If the normal portions of the liver show cellular infiltrations, amyloidosis, or other pathological processes, the tumor tissue usually exhibits these changes less markedly. The amount of connective tissue in the adenomas is always very small. All in all these growths in the mouse liver closely correspond to the typical adenomatous nodules of the human liver, except that they are not associated with cirrhosis.

Of the three tumors presenting definitely malignant characters, one showed numerous metastases of liver cell tumor tissue in the lung (Figures 1 and 3), thus establishing the true malignant character of the growth and furnishing the necessary demonstration of the truly neoplastic character of these liver tumors. The malignant tumors differ from the benign merely in the degree of deviation from the normal hepatic tissue structure; the cords of tumor cells are more often multinuclear, more irregular in arrangement, more subject to retrogressive changes and hemorrhage. Atypical nuclear forms are common, but mitotic nuclei are not often found. These features are indicated by the accompanying illustrations.

Other tumors of the liver. — In contrast to the rat, primary sarcoma was never found in the liver of mice. We have found in six mice growths of spindle cell character in

the liver, which resemble sarcomas in many respects, and which for a time were regarded as probably sarcomatous. Further study of the various tissues in these mice has, however, brought forward in each case findings which make it impossible for us to be certain that the liver growths are not granulomatous, and hence they are not included in our series of tumors of the liver. Mice are very subject to granulomatous conditions of undetermined cause, which makes a positive diagnosis of sarcomas something to be reached only with much caution. In the mice with the spindle cell growths in the liver we have found similar conditions elsewhere, in the spleen especially, often also in or about the kidney and mesenteric glands, and always there were found areas and features that seemed best interpreted as of granulomatous character. In four of them a striking feature was the plugging of some of the vessels in the spleen, liver, and lung with masses of cells of a more endothelial type. There are some features suggesting a granuloma of the spleen with metastasis in the liver. The case described by Tyzzer is rather after this order, and while the cells of this growth, which Dr. Tyzzer kindly loaned us for examination, resemble sarcoma cells in many respects, the existence of less typical growths in the spleen, and comparison with the varying features of similar cases in our own material, makes it seem probable that they are all more probably inflammatory than neoplastic. A comparison with one of the rat liver tumors loaned us by Dr. Woolley is in support of this conclusion, for the rat liver growth is unquestionably sarcoma as far as histological features can permit of diagnosis, and exhibits none of the questionable features of the mouse liver growths.

Although hemangiomas, or cavernous tissue commonly designated as such, are the commonest of growths in the liver of man and some other animals, we have not found a single instance of such tumors in mice. One liver showed a mass suggesting this character of growth, but careful examination made this diagnosis untenable. There was a nodule composed of a group of laminated thrombi enclosed by fibrous septa, resembling somewhat a thrombosed cavernous

hemangioma. This condition was evidently produced by a destruction of the liver cells of one lobule after another, absorption of the dead material and replacement by blood, various stages of the process being found outside the thrombosed area. A similar condition has been described in the liver of bovines, caused by a hyphomycete, which produces a toxic necrosis of the liver cells.

Secondary tumors are extremely rare in the livers of mice, there being in this material but three secondary carcinomas from mammary gland growths, two osteosarcomas secondary to bone tumors, and one sarcoma secondary to a mesenteric growth. This immunity of the mouse liver undoubtedly depends largely on the great rarity of abdominal cancer in mice, and the relatively slight tendency to metastasis in mouse cancer of whatever sort or origin.

SUMMARY.

Primary tumors of the liver are infrequent in mice, but twenty-eight being found in ten thousand mice of all ages dying natural deaths and carefully autopsied. There is but one other case recorded in the literature. All were liver cell adenomas, one showing malignant structure and multiple metastases in the lung, two with malignant structure without metastases, and the remainder exhibiting all degrees from probable malignancy to simple adenomas. The mouse liver does not show cirrhosis, no case of any type of cirrhosis having yet been found in all this material. No tumors of bile duct type were found. Sarcoma-like growths were found in six, but these are more probably granulomas. No hemangiomas or other tumors were found. There were three carcinomas secondary to mammary gland tumors, and three secondary sarcomas. None of the tumors seemed to be the result of cestode infection of the liver or other evident cause. The co-existence of liver tumors with other primary tumors is high, there having been multiple tumors in ten of the twenty-eight cases; in three of these both tumors were hepatic, in the other seven, different organs were involved. Only six secondary tumors were found in

the liver, three being from mammary gland carcinomas, one from a mesenteric sarcoma, and two secondary osteosarcomas.

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EXPLANATION OF PLATE XI.

1. Primary carcinoma of the liver of a mouse, with metastases in lung.—The primary tumor is seen extending downward from the remaining normal portion, which it greatly exceeds in size. At the lower edge of the left lung is a secondary growth (a) and other metastases can be seen in this lung.

2. Section of primary carcinoma shown in Figure 1.—The masses of liver cells are in alveolar form, not preserving the hepatic cord arrangement seen in the benign adenomas.

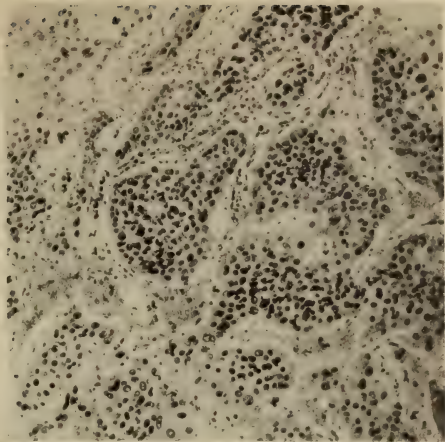
3. Section of secondary carcinoma shown in Figure 1.—A section through the tip of the left lung, including the nodule (a, Fig. 1). Some reproduction of atypical liver cords is seen here.

4. Adenoma of the liver of a mouse.—The large, rounded, partially pedunculated tumor is seen below the remaining normal liver tissue. This tumor was composed of abnormally arranged cords of liver cells, without portal systems. No metastases, and character of growth not malignant.

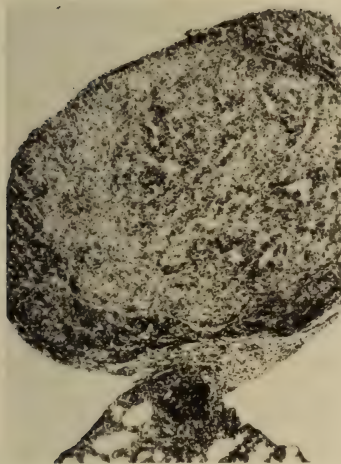
5. Section of adenoma of liver.—Showing the junction of liver and tumor, with compression atrophy of the liver cells. The tumor cells are much larger than normal liver cells; the cords are atypical in shape, size and arrangement; there are many large blood spaces, as seen at the lower edge of the field.



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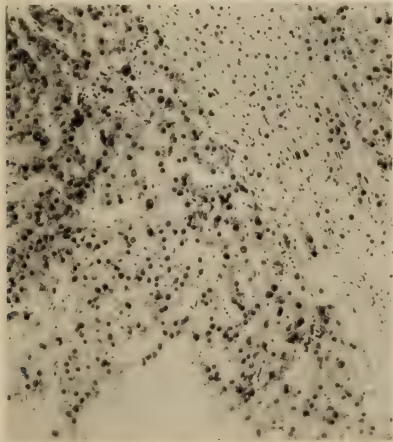
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THE INFLUENCE OF EXPOSURE TO X-RAYS UPON THE FORMATION OF ANTIBODIES.*

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The well-known selective destructive action of the Röntgen rays upon lymphadenoid tissue has suggested the employment of this agent in the study of the problem of the locus of antibody formation. Unfortunately, however, as Heineke¹ has shown, the action of X-rays, while affecting chiefly the lymphadenoid tissue, also injures the bone marrow. There is not only a destruction of leucocytes in the blood stream but a lessened production of leucocytes of all kinds by the hemopoietic organs. Hence it is not possible to produce accurately the very simple experimental conditions desired. But inasmuch as the chief effect of the X-ray is upon the lymphadenoid tissue and the chief effect of benzol is upon the bone marrow it was thought that a systematic study of the curve of the formation of antibodies in animals exposed to Röntgen rays and treated with benzol, respectively, might yield results that were worth while. This paper is concerned only with the effect of X-ray upon the production of antibodies. The effect of benzol in this relation is made the basis of a further report.

The question of the influence of Röntgen rays upon susceptibility to infection and upon the production of antibodies has been made the object of research by others, and their results are far from harmonious. Thus Quadrone² concluded that mice and guinea-pigs exposed to X-ray showed a greater resistance to infection than did normal animals. Heile³ produced a local leucocytosis in the peritoneal cavities of rabbits by the injection of aleuronat and then injected suspensions of *B. coli*. The animals so treated were divided into two groups, one of which was exposed to X-ray, the

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other not. The rabbits of the former group survived; of the latter, all died. Werner⁴ produced local abscesses by the injection of turpentine; and later injected bacteria into these abscesses. One-half of the animals were exposed to X-ray and all recovered. The others developed a progressively spreading inflammation from which they died. Heile and Werner explain their results upon the assumption that the X-rays destroyed leucocytes in the peritoneal cavity and local abscesses, respectively, setting free bactericidal substances which killed the bacteria present.

On the other hand, L  wen⁵ found that the resistance to infection of strongly X-rayed rabbits was greatly reduced and that the disintegration of leucocytes by the X-ray did not result in increasing the alexin in the blood serum. He found, further, that if the X-rays had not been applied for too long a time the injection of bacteria was followed by a rise in the number of leucocytes in the circulating blood; but that if the exposure had been for a longer period, such an injection was followed by a rapid fall in the number of leucocytes, sometimes being so extreme as to render the circulating blood aleucocytic. More recently Murphy and Ellis⁶ exposed normal and splenectomized mice to R  ntgen rays and found that they became "markedly more susceptible to bovine tuberculosis than are normal animals."

Heile³ and Werner⁴ believed that the exposure of animals to X-rays caused an increased production of alexin. McCulloch⁷ found an increase in the opsonic index for tubercle bacilli after exposure of a tuberculous focus to X-ray. He believed that by such treatment those cells which contained opsonin were killed and the opsonin set free.

Benjamin and Sluka,⁸ on the contrary, exposed rabbits to X-rays long and intensively and injected foreign serum. The production of precipitins was markedly less than in normal controls. L  wen⁴ concluded that the R  ntgen rays have no effect upon normal agglutinins but hinder the production of specific agglutinins. The animals so treated showed a pronounced leucopenia, but no change in the

erythrocytes. L  wen concluded, therefore, that the blood-making organs are concerned in the production of specific agglutinins; and that it is that part of the hemopoietic system which has to do with the formation of leucocytes, and not that from which the red cells are formed, that is responsible for the production of these antibodies. In this connection it is interesting to note that Moreschi⁹ has recently reported a series of patients with leukemia to whom anti-typhoid vaccine was given. None of these patients at any time showed agglutinins in their blood serum.

L  wen⁴ found, further, that exposure to X-rays had a much less marked effect upon the production of specific bacteriolysins.

In view of the above mentioned conflicting reports it seemed desirable to study simultaneously and systematically the relative and absolute amounts of the different antibodies in the serum of X-rayed animals after the injection of single doses of antigen. It has been shown that such an injection normally gives a typical antibody curve.¹⁰ The estimations which make possible the platting of such a curve have been made largely for agglutinins and opsonins, and these usually in isolated cases without reference to other antibodies in the serum. In those cases in which simultaneous study of two or more antibodies has been made, the different curves have not usually been found to run a parallel course. As pointed out by Hektoen,¹⁰ "This asymmetry in the curves of the antibodies educed in the same animal suggests that we are dealing with distinct substances, the production of which is dependent on similar yet not identical mechanisms." It was partly with the hope of discovering some clue to the mechanism and the locus of the production of the different antibodies that this study, and that concerned with the effect of benzol upon antibody production, was undertaken.

Medium-sized healthy rabbits were exposed to X-rays for ten to fifteen minutes a day for a period of three weeks. A "soft" tube of high penetrating power, with the current always of the same amperage, was used, but otherwise there

was no other measurement of the activity of the rays. Ordinary and differential leucocyte counts were made at frequent intervals. Except for slight loss of weight the animals did not appear to be in any way inconvenienced by the daily treatments. There was no burning of the skin and they remained quite healthy throughout the experiment. At the end of three weeks, exposure to X-ray was stopped and each animal was given an intraperitoneal injection of a suspension of typhoid bacilli killed by heating to 56° C. for thirty minutes. The reason for stopping the exposures to X-rays at the time of the injection of the antigen was to avoid any possible direct effect of the rays upon any antibodies formed and circulating in the blood. Control animals were injected with corresponding amounts of the same suspension.

Twenty-four hours before the injection of antigen was given a sample of blood was obtained for examination. Thereafter samples of blood were taken, as a rule, every other day throughout the period of the experiment. The effect of repeated bleedings tends to keep the curve of agglutinins, and possibly of other antibodies, above the level which may be considered normal for a given time (Schroeder¹¹). But in our experiments the X-rayed rabbits and the controls were bled on approximately the same days and the same amount of blood was taken from each. Hence any effect of the frequent bleedings upon the antibody curves would be the same in both groups of animals.

The results of exposure of 3 rabbits are shown in the following protocols:

Rabbit XI. — Feb. 8, 1915. Weight, 1,410 grams. Leucocytes, 14,000; polymorphonuclears, 44%; myelocytes, .5%; lymphocytes, 42.9%; basophiles, 4%; large mononuclears, 8.8%; nucleated reds, 0. Absolute number lymphocytes, 6,000 per cubic millimeter. Ten minutes exposure to X-ray.

February 9 and 10. Ten minutes daily exposure to X-ray.

February 11. Leucocytes, 13,600; polymorphonuclears, 50%; myelocytes, .5%; lymphocytes, 41.8%; basophiles, 3.8%; large mononuclears, 3.9%; nucleated reds, 0. Absolute number lymphocytes, 5,685 per cubic millimeter. Ten minutes exposure to X-ray.

February 12-16. Ten minutes exposure to X-ray daily.

February 17. Leucocytes, 6,600; polymorphonuclears, 65.6%; myelocytes, 0; lymphocytes, 18.3%; basophiles, 7.6%; large mononuclears, 8.5%; nucleated reds, 0. Absolute number lymphocytes, 1,208 per cubic millimeter.

February 18 and 19. Ten minutes exposure to X-ray daily.

February 20 to 23. Fifteen minutes exposure to X-ray daily.

February 24. Weight, 1,358 grams. Leucocytes, 7,500; red blood cells, 5,600,000.

February 25 to March 1. X-ray, 15 minutes daily.

March 2. X-ray, 15 minutes. Four cubic centimeters blood from heart.

March 3. X-ray, 15 minutes.

March 4. Weight, 1,295 grams. Leucocytes, 6,960; polymorphonuclears, 59.5%; myelocytes, 0; lymphocytes, 28%; basophiles, 0; large mononuclears, 7.5%; nucleated reds, 0. Absolute number lymphocytes, 1,950 per cubic millimeter. One and two-tenths cubic centimeters suspension of *B. typhosus* (6 24-hour agar slants in 10 cubic centimeters physiological salt solution) heated to 56° C. for 30 minutes, intraperitoneally.

March 5. Leucocytes, 14,400; polymorphonuclears, 73.7%; myelocytes, 6.3% (?); lymphocytes, 11%; basophiles, 2.1%; large mononuclears, 6.9%; nucleated reds, 1. Absolute number lymphocytes, 1,585 per cubic millimeter.

March 6. Weight, 1,275 grams. Leucocytes, 11,800; polymorphonuclears, 58.4%; myelocytes, 1.1%; lymphocytes, 31.7%; basophiles, 1.5%; large mononuclears, 7.3%; nucleated reds, 0. Four cubic centimeters blood from heart. Three to 4 cubic centimeters of blood were drawn at intervals of 1 to 4 days until March 25. The animal continued in excellent condition, without loss of weight, throughout this period. The results of the examination of this serum for antibodies will be given below.

Rabbit X2. — Feb. 8, 1915. Weight, 1,695 grams. Leucocytes, 12,800; red blood cells, 6,260,000; polymorphonuclears, 43.2%; myelocytes, 0; lymphocytes, 45.9%; basophiles, 1.7%; large mononuclears, 9.2%; nucleated reds, 0. Absolute number lymphocytes, 5,875 per cubic millimeter. X-ray, 10 minutes.

February 9 and 10. X-ray, 10 minutes daily.

February 11. Leucocytes, 8,720; polymorphonuclears, 32.5%; myelocytes, 0; lymphocytes, 60%; basophiles, 3.6%; large mononuclears, 3.9%; nucleated reds, 0. Absolute number of lymphocytes, 5,230 per cubic millimeter.

February 12 to 16. X-ray, 10 minutes daily.

February 17. Weight, 1,645 grams. Leucocytes, 4,680; polymorphonuclears, 40.3%; myelocytes, 2.5%; lymphocytes, 47.1%; basophiles, 2.6%; large mononuclears, 7.5%; nucleated reds, 1 (?). Absolute number lymphocytes, 2,200 per cubic millimeter. X-ray, 10 minutes.

February 18 and 19. X-ray, 10 minutes daily.

February 20 to 23. X-ray, 15 minutes daily.

February 24. Weight, 1,687 grams. Leucocytes, 8,200; red blood cells, 5,800,000. X-ray, 15 minutes.

February 25 to March 1. X-ray, 15 minutes daily.

March 2. X-ray, 15 minutes. Four cubic centimeters blood from heart.

March 3. X-ray, 15 minutes.

March 4. Weight, 1,675 grams. Leucocytes, 4,240; polymorphonuclears, 40%; myelocytes, 0; lymphocytes, 46.6%; basophiles, 0; large mononuclears, 13.4%; nucleated reds, 0. Absolute number lymphocytes, 1,975 per cubic millimeter. One and seven-tenths cubic centimeters suspension of *B. typhosus* (same as for Rabbit X1) intraperitoneally.

March 5. Leucocytes, 5,040; polymorphonuclears, 65.3%; myelocytes, 3.5%; lymphocytes, 24.5%; basophiles, 2.3%; large mononuclears, 4.4%; nucleated reds, 10. Absolute number lymphocytes, 1,235 per cubic millimeter.

March 6. Weight, 1,660 grams. Leucocytes, 5,920; polymorphonuclears, 50.7%; myelocytes, 3%; lymphocytes, 41.3%; basophiles, 3.0%; large mononuclears, 4.3%; nucleated reds, 1. Absolute number lymphocytes, 2,445. Three cubic centimeters blood from heart. From March 6 to March 25 3 to 4 cubic centimeters of blood were drawn from the heart at intervals of 1 to 4 days. The animal remained in good condition throughout the period of the experiment. The results of the examination of its serum for antibodies are given below.

Rabbit X3.—This animal received exactly the same treatment as Rabbits X1 and X2. It died as a result of the injection of the suspension of killed typhoid bacilli, hence its full protocol will not be given. The following facts should be presented for comparison:

February 8. Weight, 1,530 grams. Leucocytes, 9,160; red blood cells, 5,200,000; polymorphonuclears, 40.8%; lymphocytes, 49.2%. Absolute number lymphocytes, 3,735 per cubic millimeter.

February 11. Leucocytes, 10,640; polymorphonuclears, 40%; lymphocytes, 55%. Absolute number lymphocytes, 5,050 per cubic millimeter.

February 17. Weight, 1,510 grams. Leucocytes, 2,640; polymorphonuclears, 52.7%; lymphocytes, 39.4%. Absolute number of lymphocytes, 1,040 per cubic millimeter.

February 24. Weight, 1,510 grams. Leucocytes, 4,400.

March 4. Weight, 1,415 grams. Leucocytes, 3,840; polymorphonuclears, 46%; lymphocytes, 40.8%. Absolute number lymphocytes, 1,565 per cubic millimeter. One and four-tenths cubic centimeters suspension *B. typhosus* (same as for X1 and X2) intraperitoneally.

March 5. Found dead in cage.

Autopsy: Inguinal and axillary lymphatic glands about 2 x 3 millimeters. Spleen of normal size and appearance. Liver and kidneys show no gross change. Peritoneal cavity contains 5 to 10 cubic centimeters of slightly turbid fluid, but the peritoneum is everywhere smooth and glistening. Stomach and intestines greatly distended with food and gas. Peyer's patches not distinctly visible. Bone marrow grayish red in color. Lungs normal. Heart shows former needle puncture, but the pericardial cavity contains no blood or clots.

A consideration of these protocols reveals the following facts:

1. There was a steady decline in the number of leucocytes in the peripheral blood during the period of exposure to X-rays. This fall affected chiefly the lymphocytes but the polymorphonuclears were also, but to a less degree, reduced in number. That the diminution in the number of leucocytes was due to actual destruction of the individual elements and not to their accumulation in the capillaries of

the viscera, is shown by the fact that damaged leucocytes and leucocytic débris were seen in the stained smears although very much less abundant than in the smears of blood from rabbits injected with benzol.

2. The exposures had no effect upon the red blood cells themselves nor upon the erythrocytogenic power of the bone marrow. There were no poikilocytosis, no stippling, and no nucleated reds to be seen before the injection of the antigen.

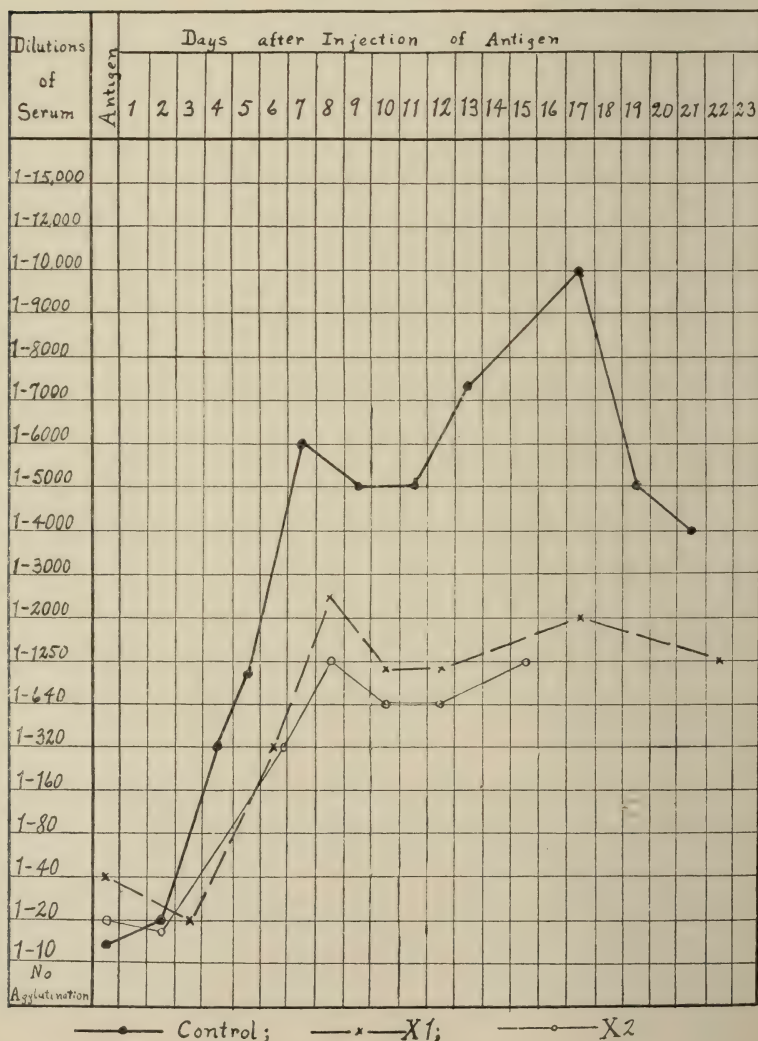
3. The injection of the antigen had a most striking effect: (a) In Rabbit X1 there was a sharp rise in the number of leucocytes affecting, at first, only the polymorphonuclears and accompanied by a slight fall in the absolute and relative number of lymphocytes. Later there was an increase in the number of lymphocytes and a fall to about normal in the total number of leucocytes. The differential count was also practically normal. (b) In Rabbit X2 there was at first a relative lymphocytosis which appeared to increase under the influence of the first few exposures to X-rays. Later, there was a marked fall in the total leucocyte count and an absolute and relative diminution in the number of lymphocytes. The injection of antigen was followed by a very slight increase in the total number of leucocytes, which still remained below normal. The effect upon the bone marrow was shown by the relatively large number of nucleated red cells seen in the smears. (c) Rabbit X3, which showed the lowest total leucocyte count (3,840 per cubic millimeter), did not survive the same dose, in comparison to body weight, that had been received by the other two animals.

Agglutinins. — The macroscopic method of agglutination was used throughout these experiments. Chart I. shows characteristic agglutinin curves of two X-ray rabbits and a composite curve of three normal animals which had been injected with corresponding doses of the same suspensions of killed typhoid bacilli with which the X-ray rabbits had been injected. The lowest titer of the serum of any of the

control animals was always higher than the highest titer of the serum of the X-ray rabbits on the corresponding days. It is seen from this chart that the exposure to X-rays appreciably lowered the power of these animals to produce agglutinins.

CHART I.

Showing the Effect of X-rays upon the Production of Agglutinins for *B. typhosus*.



Bacteriolysins. — The attempt to study the production of lysins for *B. typhosus* in X-rayed rabbits did not prove entirely satisfactory. Hence we will only state briefly the results of the work without presenting all the details of the experiments. The technic described by Neisser¹² was used throughout these experiments. The same sera of the same X-rayed and control rabbits were used for the study of bacteriolysins as were used in the estimation of agglutinins.

Our chief difficulty lay in the impossibility of determining the bacteriolytic power of the normal rabbit serum used as complement. We used uniformly .5 cubic centimeter of a 1-10 dilution of normal rabbit serum in two cubic centimeters of mixture, making a final dilution of complement of 1-40. Not infrequently we would find complete bacteriolysis in all dilutions of the inactivated immune serum employed. Repeating the experiment the following day with the same immune serum, kept on ice in the meantime, but using the serum of a different rabbit for complement it became evident that the previous results were due largely to the high content of the complementary serum in bacteriolytic amboceptor.

However, a study of the results of many experiments, often repeated two or three times with different complementary sera, justifies, we believe, the following statements: After the injection of X-rayed rabbits with a single large dose of killed typhoid bacilli there is a definite increase in the typholytic power of the serum of such animals. The bacteriolysin content of the serum of the X-rayed rabbits does not differ as much from the bacteriolysin content of the normal controls as the hemolytic power of the serum of immunized benzol rabbits differed from the hemolytic power of the corresponding controls.

Opsonins. — The dilution method of determining the opsonic content of serum was employed in these studies. Capillary pipettes were used for mixing the serum dilutions and the leucocytic and bacillary suspensions according to the well-known technic of Wright. The pipettes containing

the mixtures were incubated for twenty minutes and smears made after again thoroughly mixing the ingredients. The smears were stained with carbol-thionin. The results of this series of estimations are shown in Table I. In this chart the dilutions of serum given are the final dilutions in the pipette after being mixed with equal volumes of the suspensions of bacilli and leucocytes. It would seem from these results that as compared with normal control animals, exposure to X-ray did not materially affect the ability of the rabbit to produce opsonins for the typhoid bacillus. There was no evidence of any increased production of opsonins as found by McCulloch in his investigation of the opsonic index for *B. tuberculosis* of the serum of tuberculous patients exposed to X-ray.

Complement fixation. — The determination of the complement fixing power of the serum of X-rayed rabbits after the injection of killed typhoid bacilli was thought to be of interest in view of recent work which seemed to indicate that the lymphocytes may have something to do with the formation of the bodies responsible for the binding of complement. Wassermann and Lange¹³ concluded that at least one source of complement fixing substances in the cerebrospinal fluid is the lymphocytes which it contains. They found that all such fluids which gave a positive Wassermann reaction contained an increased number of lymphocytes; and that autolysis of these cells increased, sometimes very greatly, the complement-fixing power of the fluid. Tsurumi and Kohda¹⁴ found complement fixing substances in the spleen twenty hours after injection of *B. typhosus*, at which time these bodies could not be found in other organs.

In our experiments we used an antigen made from the same strain of *B. typhosus* as was used in immunizing the animals whose serum was being tested. Suspensions in physiological salt solution of twenty-four-hour agar slant cultures were shaken vigorously with glass beads to break up clumps and heated to 60° C. for two hours. The suspensions had a turbidity a little less than that of an eighteen-hour

plain broth culture of the same organism. An anti-human hemolytic system was used.

The tests were carried out as follows: .5 cubic centimeter of antigen and .5 cubic centimeter of a 1-10 dilution of normal guinea-pig serum were placed in small test-tubes. To each of these was added from a capillary pipette two drops of the serum of one of the X-rayed or control rabbits, respectively. The tubes were then placed in the incubator for one hour. At the end of this time there was added to each tube .5 cubic centimeter of a five per cent suspension human erythrocytes and .5 cubic centimeter of a dilution of anti-human immune serum equal to twice the dose previously found to be sufficient to cause complete hemolysis of .5 cubic centimeter of the same suspension of human red blood cells in the presence of .5 cubic centimeter of a 1-10 dilution of the same complement as was used in the final test. The tubes were placed in the incubator for an hour and a half and then set aside in the refrigerator for twelve hours, when the final results were recorded. Control tests showed that in the amounts used neither the antigen nor the immune sera alone possessed any complement fixing or anti-hemolytic powers.

The results of the study of complement-fixing bodies in the same sera examined simultaneously for agglutinins, lysins, and opsonins are shown in Table II. In this table the minus sign indicates that there was complete hemolysis and therefore no complement fixation. Four pluses signify complete absence of hemolysis and therefore complete complement fixation. One, two, and three pluses indicate decreasing grades of hemolysis and therefore increasing degrees of binding of complement.

From these experiments it does not appear that exposure to X-rays, as here carried out, hinders the formation of complement-binding substances when these animals are immediately thereafter injected with killed typhoid bacilli. It is interesting to note that the serum of one of the control animals did not at any time show any complement-fixing power.

SUMMARY.

1. These experiments were carried out with two objects in mind: (1) It was thought that, in view of the fairly specific destructive action of X-rays upon lymphadenoid tissue, a simultaneous and systematic study of the production of various antibodies in X-rayed animals (rabbits) after the injection of large single doses of antigen might yield some information concerning the locus and mechanism of antibody formation. (2) It was thought, further, that a study of the formation of the different antibodies in animals repeatedly exposed to X-rays might help to explain the greatly increased susceptibility to infection which has been found to exist in such animals.

2. Rabbits were the animals used. These were exposed to X-rays for ten to fifteen minutes daily for a period of three weeks. The exposures were stopped and a single large dose of killed typhoid bacilli was given intraperitoneally to each animal. The immediate effect, *i.e.*, within the first forty-eight hours, seems to indicate a lowered resistance on the part of the X-rayed animals. The fact that our results are not so striking as might have been expected from the published reports of decrease of resistance to infection in mice exposed to X-ray may be due in part to the fact that our experiments were carried out entirely on rabbits.

3. The effect of exposure to X-rays upon the production of different antibodies may be summarized as follows:

(*a.*) The formation of agglutinins is appreciably lowered, although not so markedly as in the case of rabbits injected with benzol.

(*b.*) The attempt to study bacteriolysins for typhoid bacilli was less satisfactory than in the case of other antibodies. Our results do not warrant a positive statement, but the indications are that the production of bacteriolysins for *B. typhosus* in our animals was not greatly interfered with by exposure to X-rays.

(*c.*) The opsonic content and the complement-fixing

power of the serum of X-rayed rabbits did not differ appreciably from that of the control animals.

[For the exposures to X-rays we are indebted to Miss A. H. Brindley of the X-ray Laboratory of Wesley Memorial Hospital.]

TABLE I.
Showing the effects of exposure to X-ray upon the production of opsonins for B. typhosus.

Date.	Animal.	Dilutions of Serum. — Phagocytosis.						
		1-3.	1-15.	1-30.	1-60.	1-120.	1-240.	1-480.
March 4. Anti- gen Injected.	X-ray 1.	+++	+	—				
	X-ray 2.	+++	+	—				
	Control 1.	+++	+	—				
	Control 2.	+++	+	—				
	Control 3.	++	—	—				
March 6	X-ray 1.	+++	+++	+++	—	—		
	X-ray 2.	—	—	—	—	—		
	Control 1.	+++	+++	+++	—	—		
	Control 2.	+++	+++	+++	+++	—		
	Control 3.	+++	+++	+++	+++	—		
March 9	X-ray 1.	+++	+++	+++	+++	+++	+	—
	X-ray 2.	+++	+++	+++	+++	+	—	—
	Control 1.	+++	+++	+++	+++	+	—	—
	Control 2.	+++	+++	+++	+++	+	—	—
	Control 3.	+++	+++	+++	+++	—	—	—
March 15	X-ray 1.	+++	+++	+++	+++	+	—	—
	X-ray 2.	+++	+++	+++	+++	+++	+++	+
	Control 1.	+++	+++	+++	+++	+++	+++	+++
	Control 2.	+++	+++	+++	+++	+++	+++	+
	Control 3.							

+++ Vigorous phagocytosis. — No phagocytosis.

TABLE II.

Showing the effects of exposure to X-ray upon the production of complement-fixing substances for B. typhosus.

Date.	Animal. — Complement-fixation.			
	X-ray 1.	X-ray 2.	Control 1.	Control 2.
March 4	—	—	—	—
March 6	—	—	—	—
March 9	—	—	++	—
March 11	++	++	++++	—
March 13	++++	++++	++++	—
March 15	+++	+	++++	—
March 25	++++		++++	—

++++ Complete complement fixation. (No hemolysis.)

+++ Almost complete complement fixation. (Slight hemolysis.)

++ Partial complement fixation. (Partial hemolysis.)

+ Slight complement fixation. (Almost complete hemolysis.)

— No complement fixation. (Complete hemolysis.)

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THE EFFECT OF INJECTIONS OF BENZOL UPON THE PRODUCTION OF ANTIBODIES.*

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The evidence that the blood-making organs are essentially concerned in the production of antibodies is thoroughly convincing. But, as pointed out by Hektoen,¹ the asymmetry in the curves of the different antibodies educed in the same animal indicates that the mechanisms of the production of the various antibodies are not identical. It seemed to us that a study of the curves of the different antibodies in animals exposed to X-rays, which exert a destructive action chiefly upon the lymphadenoid tissue, and in animals treated with benzol, which affects chiefly the bone marrow, might throw some light upon the difference in the mechanism of the formation of the various immune bodies. The influence of X-rays has been made the basis of a separate report.² The results following injections of benzol are here presented.

The effects upon resistance to infection and the production of antibodies of other toxic substances which affect the hemopoietic organs less specifically than does benzol have been investigated. But, so far as we are aware, there are no published reports of systematic studies of the production of different antibodies in animals injected with benzol.

Madsen and Talquist³ found that pyrogallol and pyrocin given in increasing doses in the third stage of antibody formation caused a rise in the curve of antilysins for vibriolysin and staphylokin. Malnikowa and Wersilowa⁴ studied the production of agglutinins for *B. typhosus* in animals injected with hydroxylamin and phenylhydrazin. These substances caused a destruction of red blood corpuscles which was associated with a rapid fall in the agglutinin titer of the serum. The subsequent regeneration of erythrocytes was not accompanied by a rise in the agglutinin content of the

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blood. The fact, however, that injection of nucleins, which reduced the number of red blood corpuscles and caused a leucocytosis, was accompanied by an increase in the production of agglutinins above that in control animals, led these authors to consider it probable that the depressing influence of hydroxylamin and phenylhydrazin was due to their effect upon the leucocytes instead of upon the erythrocytes.

Lippmann⁵ studied the effect upon antibody production of substances which irritate the bone marrow ("Knochenmarksreizmittel"), using for this purpose Thorium X, salvarsan, and some other substances. Thorium X caused a rise in the curve of agglutinins for typhoid bacilli without the further injection of antigen. Both Thorium X and salvarsan in doses sufficient to "irritate the bone marrow" caused mice to survive many times the minimum fatal dose of pneumococci. Amboceptor production, however, was not influenced by these bone marrow irritants.

Schiff⁶ found that small doses of benzol increased, while large doses decreased, the sensitiveness of animals sensitized to foreign proteid. Kline and Winternitz⁷ found that rabbits which had been injected with benzol showed a markedly lowered resistance to intratracheal injections of pneumococci.

While our work was in progress Hektoen⁸ reported the results of somewhat similar experiments to the Chicago Pathological Society (May, 1915). He used defibrinated sheep blood as antigen and studied the production of precipitins and hemolysins in rabbits treated with benzol. Doses of benzol in the proportion of one cubic centimeter per kilo caused a marked fall in the leucocyte counts, and the production of these antibodies was greatly reduced or even entirely suppressed.

In our work we were greatly hampered by the high death rate among the animals being injected with benzol. They succumbed very readily to spontaneous infections, and when the leucocyte counts became very low (*i.e.*, 1,000 or less per cubic millimeter) the animals died without any definite evidence of infection. These disconcerting results were also mentioned by Hektoen.

The dose employed in almost all cases was one cubic centimeter of benzol (Merck) dissolved in two cubic centimeters of olive oil per kilogram of body weight. The injections were invariably given subcutaneously. The reaction of the different animals to the injections was quite variable. Some responded promptly with a rapidly progressing leucopenia. These did not develop a leucocytosis after the injection of the antigen if the number of white blood cells had fallen below a certain point, about two thousand per cubic millimeter. In other animals it was almost impossible, even with larger doses, to produce a very marked leucopenia. In these animals the injection of an antigen, especially killed bacteria, was followed by a rapidly developing and very pronounced leucocytosis—in one animal, almost forty thousand leucocytes per cubic millimeter.

For antigen we used washed dog-blood corpuscles and killed typhoid bacilli. The study of hemolysins instead of bacteriolysins was due to the greater accuracy with which the hemolytic titer of a serum can be measured. Dog blood was used partly because it was more conveniently available than any other and partly because we have not found any natural hemolysins in rabbit serum for dog corpuscles. The same strain of *B. typhosus* was used in all of our experiments.

The following five protocols are given in detail because they are characteristic of many other experiments and because they represent animals which survived for a sufficient period of time to permit the platting of a curve of some length.

Rabbit B 20. — May 4, 1915. Weight, 1,380 grams. Leucocytes, 8,000 per cubic millimeter. Six cubic centimeters olive-oil-benzol mixture (equivalent to 2 cubic centimeters benzol).

May 5. Six cubic centimeters olive-oil-benzol mixture (hereafter designated "O.B. mixture").

May 6. Weight, 1,370 grams. Leucocytes, 5,840; polymorphonuclears, 46.1%; myelocytes, 0; lymphocytes, 48.5%; basophiles, 1.2%; large mononuclears, 4.2%; nucleated reds, 0. Absolute number polymorphonuclears, 2,735; lymphocytes, 2,830. Much leucocytic debris and many blood platelets. Red

cells show no important change. Four cubic centimeters blood drawn from heart. Four cubic centimeters O.B. mixture injected subcutaneously. Fifteen cubic centimeters suspension of dog corpuscles intraperitoneally.

May 7. Five cubic centimeters O.B. mixture.

May 8. Leucocytes, 1,120. Four cubic centimeters blood from heart. Four cubic centimeters O.B. mixture subcutaneously. Polymorphonuclears, 67.2% (?); lymphocytes, 32.8% (?); large mononuclears, 0. Absolute number of polymorphonuclears, 750; lymphocytes, 370. Only 61 leucocytes counted in the entire smear. Much leucocytic debris. Red cells showed no noteworthy change.

May 10. Four cubic centimeters blood from heart.

May 12. Leucocytes, 3,440. Animal has snuffles. Four cubic centimeters blood from heart. Polymorphonuclears, 58.5%; basophiles, .6%; lymphocytes, 34.5%; unidentified, .5%; large mononuclears, 5.9%; nucleated reds, 1. Absolute number polymorphonuclears, 2,010; lymphocytes, 1,185. One hundred and seventy-one leucocytes counted. Many leucocytes so changed that accurate classification was impossible. Except for one nucleated red, the erythrocytes showed little or no change.

May 14. Four cubic centimeters blood from heart.

May 15. Five cubic centimeters O.B. mixture.

May 18. Four cubic centimeters blood from heart. Four cubic centimeters O.B. mixture. Weight, 1,160 grams.

May 19. Leucocytes, 1,600. Animal in poor condition. Polymorphonuclears, 39.2%; basophiles, .8%; lymphocytes, 50.3%; unidentified, 6.5%; large mononuclears, 3.2%; nucleated reds, 9. Absolute number polymorphonuclears, 625; lymphocytes, 800. One hundred and fifty-three leucocytes counted. Much leucocytic debris. Many leucocytes undergoing disintegration so that accurate identification was impossible. In addition to 9 nucleated red cells counted there was polychromatophilia and poikilocytosis but no stippling.

May 20. Found dead in cage.

Autopsy: Heart shows no damage as a result of bleedings. Pericardium contains no blood. Lungs normal. Liver paler than normal. Kidneys pale; cortex slightly swollen and moist. Adrenals darker in color and redder than normal. Bone marrow grayish-red with numerous pin-point-sized dark red spots. Much unabsorbed oil and benzol under skin.

Rabbit B 21. — May 4, 1915. Weight, 1,530 grams. Six cubic centimeters O.B. mixture.

May 5. Six cubic centimeters O.B. mixture.

May 6. Leucocytes, 12,200. Weight, 1,525 grams. Polymorphonuclears, 51.4%; myelocytes, (?) .9%; lymphocytes, 41.8%; unidentified, 1.4%; large mononuclears, 4.5%; nucleated reds, 0. Absolute number polymorphonuclears, 6,210; lymphocytes, 5,120. Red cells show polychromatophilia and stippling. Five cubic centimeters O.B. mixture. Four cubic centimeters blood from heart. Fifteen cubic centimeters suspension of dog blood.

May 7. Four cubic centimeters O.B. mixture.

May 8. Leucocytes, 12,320. Four cubic centimeters blood from heart. Five cubic centimeters O.B. mixture. Polymorphonuclears, 32.8%; unidentified, 4.5%; lymphocytes, 62.3%; nucleated reds, 8; large mononuclears, .4%. Absolute number polymorphonuclears, 4,075; lymphocytes, 7,675. Much leucocytic

débris and many blood platelets (?). Many stippled cells. A few megaloblasts. Some polychromatophilia and poikilocytosis.

May 10. Four cubic centimeters blood from heart.

May 12. Leucocytes, 12,080. Four cubic centimeters blood from heart. Polymorphonuclears, 41%; unidentified, 2.1%; lymphocytes, 51.2%; nucleated reds, 0; large mononuclears, 5.7%. Absolute number polymorphonuclears, 4,950; lymphocytes, 6,160. Red cells showed stippling and chromatophilia. Less leucocytic débris than on May 8.

May 14. Four cubic centimeters blood from heart.

May 15. Five cubic centimeters O.B. mixture.

May 18. Four cubic centimeters blood from heart. Four cubic centimeters O.B. mixture. Weight, 1,310 grams.

May 19. Found dead. No autopsy on account of advanced stage of decomposition.

Rabbit B 22. — May 5, 1915. Six cubic centimeters O.B. mixture.

May 6. Weight, 2,130 grams. Leucocytes, 7,960. Six cubic centimeters O.B. mixture. Polymorphonuclears, 40.8%; basophiles, 2%; lymphocytes, 51.6%; unidentified, 1.2%; large mononuclears, 4.4%; nucleated reds, 0. Absolute number polymorphonuclears, 3,260; lymphocytes, 4,060. Twenty cubic centimeters suspension dog corpuscles intraperitoneally.

May 7. Five and five-tenths cubic centimeters O.B. mixture.

May 8. Leucocytes, 1,480. Five cubic centimeters blood from heart. Polymorphonuclears, 29%; unidentified, 4.2%; lymphocytes, 64%; nucleated reds, 0; large mononuclears, 2.8%. Absolute number polymorphonuclears, 430; lymphocytes, 950. Many leucocytes undergoing disintegration. Much leucocytic débris. Red cells show no noteworthy changes.

May 10. Four cubic centimeters blood from heart.

May 12. Leucocytes, 2,120. Four cubic centimeters blood from heart. Five cubic centimeters O.B. mixture. Polymorphonuclears, 21.8%; basophiles, 1.6%; lymphocytes, 70%; unidentified, 4.4%; large mononuclears, 1.6%; nucleated reds, 0. Absolute number polymorphonuclears, 465; lymphocytes, 1,485. Condition of red cells practically normal. Leucocytic disintegration slightly less than on May 8.

May 14. Four cubic centimeters blood from heart.

May 15. Six cubic centimeters O.B. mixture.

May 18. Four cubic centimeters blood from heart. Six cubic centimeters O.B. mixture. Weight, 1,630 grams.

May 19. Leucocytes, 3,960. Animal appears ill. Polymorphonuclears, 27.5%; myelocytes, .4%; lymphocytes, 60.1%; basophiles, .5%; large mononuclears, 5.5%; unidentified, 6%; nucleated reds, 16. Absolute number polymorphonuclears, 1,070; lymphocytes, 2,375. Red cells show marked changes. Of the 16 nucleated reds 4 were megaloblasts. Irregularity in amount of hemoglobin in different cells. Much polychromatophilia and poikilocytosis.

May 21. Four cubic centimeters blood from heart. General condition of animal much improved.

May 26. Four cubic centimeters blood from heart. Animal in good condition.

June 15. Animal in good condition. Weight, 1,980 grams.

Rabbit B 23. — May 5, 1915. Six cubic centimeters O.B. mixture.

May 6. Weight, 2,230 grams. Leucocytes, 5,480. Seven cubic centimeters O.B. mixture. Polymorphonuclears, 32%; nucleated reds, 0; lymphocytes, 65.7%; large mononuclears, 1.3%. Absolute number polymorphonuclears, 1,750; lymphocytes, 3,615. Red cells showed no morphological changes. One 24-hour agar slants of *B. typhosus* suspended in 3 cubic centimeters physiological salt solution, heated to 56° C. for 30 minutes and injected intraperitoneally.

May 7. Five and five-tenths cubic centimeters O.B. mixture.

May 8. Leucocytes 3,200. Four cubic centimeters blood from heart. Five cubic centimeters O.B. mixture. Polymorphonuclears, 20.4%; unidentified, 5.6%; lymphocytes, 72%; nucleated reds, 0; large mononuclears, 2%. Absolute number polymorphonuclears, 640; lymphocytes, 2,300. Many disintegrating leucocytes. Red cells show nothing noteworthy.

May 10. Four cubic centimeters blood from heart.

May 12. Leucocytes, 7,360. Four cubic centimeters blood from heart. Six cubic centimeters O.B. mixture. Polymorphonuclears, 14.6%; basophiles, 1.2%; lymphocytes, 80.8%; unidentified, 2.4%; large mononuclears, 1.7%; nucleated reds, 1. Absolute number polymorphonuclears, 1,100; lymphocytes, 5,960. Many disintegrating leucocytes. Red cells show polychromatophilia and poikilocytosis.

May 14. Four cubic centimeters blood from heart.

May 15. Six cubic centimeters O.B. mixture.

May 18. Weight, 2,020 grams. Four cubic centimeters blood from heart. Six cubic centimeters O.B. mixture.

May 19. Leucocytes, 3,640. In very poor condition. Polymorphonuclears, 40.8%; basophiles, 1.4%; lymphocytes, 52.3%; unidentified, 4.1%; large mononuclears, 1.4%; nucleated reds, 85. Absolute number polymorphonuclears, 1,490; lymphocytes, 1,890. Enormous number nucleated red cells. Other erythrocytes showed marked polychromatophilia, poikilocytosis and stippling. Much leucocytic debris.

May 20. Found dead in cage. Autopsy: Pericardial cavity distended with blood and a fibrinous clot adherent to the visceral pericardium. Atelectasis of the lungs adjacent to the heart. Liver and kidneys pale and normal markings not distinct. Spleen slightly enlarged. Mediastinal lymph glands enlarged. Bone marrow of femur very dark red in color. Histologic examination showed: fibrinous pericarditis; a mild grade of glomerulo-nephritis.

Rabbit B 24. — May 5, 1915. Six cubic centimeters O.B. mixture.

May 6. Weight, 1,675 grams. Leucocytes, 6,880. Six cubic centimeters O.B. mixture. Four cubic centimeters blood from heart. Polymorphonuclears, 41.3%; unidentified, 1.3%; lymphocytes, 56.8%; nucleated reds, 1. Large mononuclears, .6%. Absolute number polymorphonuclears, 2,820; lymphocytes, 3,920. Some disintegrating leucocytes and leucocytic debris. Red cells showed polychromatophilia, poikilocytosis and stippling. One 24-hour agar slant culture of *B. typhosus* suspended in 3 cubic centimeters salt solution, heated to 56° C. for 30 minutes and injected peritoneally.

May 7. Five and five-tenths cubic centimeters O.B. mixture.

May 8. Leucocytes, 1,600. Five cubic centimeters blood from heart. Polymorphonuclears, 30%; basophiles, .8%; lymphocytes, 58%; unidentified, 4.5%;

large mononuclears, 6.7%; nucleated reds, 1. Absolute number polymorphonuclears, 480; lymphocytes, 930. Very many disintegrating leucocytes and much leucocytic debris. Red cells showed some poikilocytosis and much polychromatophilia and stippling.

May 10. Four cubic centimeters blood from heart.

May 12. Leucocytes, 2,840. Four cubic centimeters blood from heart. Four cubic centimeters O.B. mixture. Polymorphonuclears, 33.3%; myelocytes, .4%; lymphocytes, 60.8%; basophiles, 1.6%; large mononuclears, 2.9%; unidentified, 1%; nucleated reds, 0. Absolute number polymorphonuclears, 940; lymphocytes, 1,730. Red cells showed some polychromatophilia. Less disintegration of leucocytes than on May 8.

May 14. Four cubic centimeters blood from heart.

May 15. Five cubic centimeters O.B. mixture.

May 18. Weight, 1,555 grams. Four cubic centimeters blood from heart. Five cubic centimeters O.B. mixture.

May 19. Leucocytes, 5,120. Animal appears ill. Polymorphonuclears, 40.5%; myelocytes, .5%; lymphocytes, 46.5; basophiles, 2.5; large mononuclears, 7.5%; unidentified, 2.5; nucleated reds, 12. Absolute number polymorphonuclears, 2,050; lymphocytes, 2,355. Much leucocytic disintegration and leucocytic debris. Red cells show polychromatophilia and some poikilocytosis and stippling. One megaloblast.

May 21. Four cubic centimeters blood from heart.

May 24. Found dead in cage. Autopsy: Marked fibrinopurulent pleurisy. Bone marrow dark red. Otherwise no noteworthy gross changes.

In connection with these protocols attention should be called to the following facts:

1. There is a marked individual variation in the effect of benzol upon the leucocytes in different animals. Rabbits B 20, B 22, and B 24 showed an early, marked, and persistent diminution in the number of leucocytes in the circulating blood. B 23 showed a less pronounced fall in the number of leucocytes and a definite tendency to recovery if the injections of benzol were stopped for two or three days. In B 21 the leucocytes were above ten thousand at every count made, although this animal had received the same treatment as the others.

2. There is an equally marked individual variation in the effect upon the erythrocytes. Rabbits B 20, B 22, and B 24, which showed the earliest and most marked diminution in the number of leucocytes, showed almost no change in the red cells until late in the period of the experiment and even then in a less degree than in the other two animals. In B 21,

on the other hand, which continued throughout the experiment to show a high leucocyte count, there was present very early evidence of serious damage to the erythrocytogenic organs, as shown by the presence of many nucleated and stippled red cells. In B 23 the effect of the injections upon the leucocytes was much less than in B 20, B 22, and B 24, but greater than in B 21, while the damage to the red cells was so great that on the day before the animal was found dead, eighty-five nucleated red cells were observed while counting one hundred and forty-seven leucocytes.

3. The most pronounced destructive effect of benzol is upon the polymorphonuclears, but the lymphocytes are also, though to a less degree, affected.

4. Although not especially evident in any of the protocols quoted here, in some other animals in this series there was an increase of basophiles, sometimes to six per cent, whenever there was a rise in the leucocyte count upon temporarily stopping the injections of benzol.

Lysins. — The production of hemolysins for dog corpuscles was studied in a number of animals, but in only three cases did the rabbits survive long enough to permit the platting of antibody curves of sufficient length to show the first two or three phases of such a curve. In all the animals of the series the antibody curves ran a course, during the period of survival, quite similar to those found for the three given here in detail.

The dog blood was drawn into one per cent potassium citrate solution, centrifugalized, suspended in physiological salt solution and again centrifugalized. The supernatant fluid was pipetted off and the remaining very heavy suspension of dog corpuscles injected as antigen.

Every two to four days four cubic centimeters of blood were taken with a fine needle from the heart of each rabbit while under light ether anesthesia. The serum was allowed to separate in the ice box and was then pipetted away from the clot. It was inactivated by heating to 56° C. for thirty minutes. One cubic centimeter of each of the desired dilutions of the serum was placed in test-tubes and to this

In Chart I. will be seen the hemolysin curves of three rabbits injected with benzol and a composite curve representing the hemolytic power of control rabbits injected with the doses of antigen (dog corpuscles) in the same proportion to body weight as had been given to the benzol rabbits. From this Chart it is evident that the power to produce hemolysins for dog corpuscles was very greatly reduced by the injection of benzol in the doses used. The titer of the serum of the benzol rabbits ranged from one-sixteenth to one-fourth that of the control sera on the corresponding days.

The results of these experiments, as shown in Chart I., do not solve the problem of the relation of antibody formation to the erythrocytogenic and leucocyctogenic powers, respectively, of an animal; but there are certain facts to which attention may be called. From Chart I., and the corresponding protocols, it would appear that that part of the blood-making system which has to do with the production of erythrocytes may be a factor of some importance in the formation of hemolysins. Thus, Rabbit B 21 showed a constantly high leucocyte count, while the leucocytes of B 20 and B 22 very quickly fell to a little more than one thousand per cubic millimeter and continued to fluctuate between this number and three thousand nine hundred. Yet the hemolysin curves of all three animals ran approximately a parallel course. In all three animals, however, the injections of benzol had a marked effect upon the erythrocytogenic power of the bone marrow as shown by the presence of stippled and nucleated red cells. Furthermore, the serum of Rabbit B 22, on the twelfth day after the injection of the antigen, showed a titer of 1 to 64. A blood count on the following day showed nearly four thousand leucocytes per cubic millimeter (more than double the previous count), and very many nucleated red cells, some of them megaloblasts. Two days later (the fifteenth) the highest dilution of this animal's serum which caused lysis of dog corpuscles was 1 to 16.

That the presence of nucleated reds, stippled cells, etc., was not due to the frequent bleedings is evident from the

facts (1) that Rabbit B 21 showed very pronounced changes in the red cells in a blood smear taken just before the second bleeding; (2) that other animals in this series showed similar changes before they had been bled at all; and (3) that the control animals, which lost the same amount of blood, showed scarcely any changes in the erythrocytes even after six to eight bleedings. Hence we felt justified in assuming that the presence of poikilocytes, stippled cells, and nucleated red cells was due to the effect of the injections of benzol.

Agglutinins. — In the study of the production of agglutinins in rabbits injected with benzol, suspensions of killed typhoid bacilli were used as antigen, and the ordinary macroscopic method of determining the agglutinating power of the serum was employed. The agglutinin curves of Rabbits B 23 and B 24, whose protocols are given above, together with curves of control animals, are shown in Chart II. We may call attention to the following facts in this chart and the corresponding protocols:

1. The injection of benzol in doses of one cubic centimeter per kilo of body weight very materially reduces the power of an animal to produce agglutinins for *B. typhosus*. The agglutinative power of the serum of the benzol rabbits varied from one-half to about one-tenth that of the control rabbits on the corresponding days. The reduction in power to produce agglutinins is not, therefore, quite so great, proportionally, as the reduction in the power to produce hemolysins. The injection of benzol caused a greater reduction in power to produce agglutinins than did exposure to X-rays. The agglutinin titer of the serum of the X-rayed rabbits was about one-fifth that of the controls.

2. Rabbit B 24 showed a lower leucocyte count and a lower agglutinating titer of its serum than did B 23.

3. After the sixth day the sera of both animals exhibited a very marked fall in agglutinin content. In the case of B 23 this was accompanied by a fall in the leucocyte count, while in the case of B 24 it was associated with a rise in the number of leucocytes. Blood smears from both animals at this

Opsonins. — The sera of Rabbits B 23 and B 24 and the corresponding controls were tested for opsonins. The sera were kept at approximately 10° to 12° C. for twenty-four to thirty-six hours to allow deterioration of the complement to take place. This was to avoid the action of the normal and immune lysins for typhoid bacilli. Dilutions of each serum were made and equal parts of each dilution, suspension of typhoid bacilli, and suspension of washed human leucocytes were mixed in small pipettes and incubated for twenty minutes. The ingredients of the pipettes were again mixed, and smears made and stained with carbolthionin. No attempt was made to determine the opsonic or phagocytic index. The highest dilution in which phagocytosis took place was considered to represent the relative opsonin content of the respective serum.

The results are shown in Table I., in which the serum dilutions at the head of each column represent the final dilution of the serum in the pipette. From this table it is seen that the injection of benzol diminished the power of the animal to produce opsonins, but not so greatly as it reduced the power to form hemolysins or even agglutinins.

TABLE I.

Showing the effect of injections of benzol upon the production of opsonins for B. typhosus.

Date.	Animal.	Dilutions of Serum. — Phagocytosis.						
		1-3.	1-15.	1-30.	1-60.	1-120.	1-240.	1-480.
May 6. Anti- gen injected.	B 23.	+	—	—	—	—	—	—
	B 24.	+(?)	—	—	—	—	—	—
	Control 5.	+	+	—	—	—	—	—
	Control 7.	+++	+	—	—	—	—	—
May 8.	B 23.	—	—	—	—	—	—	—
	B 24.	+(?)	—	—	—	—	—	—
	Control 5.	+++	+++	+	—	—	—	—
	Control 7.	+++	+++	+++	+	—	—	—
May 10	B 23.	+++	—	—	—	—	—	—
	B 24.	++	—	—	—	—	—	—
	Control 5.	+++	+++	+++	+++	++	—	—
	Control 7.	+++	+++	+++	+++	+	—	—
May 12	B 23.	+++	+++	+	—	—	—	—
	B 24.	+++	++	+	—	—	—	—
	Control 5.	+++	+++	+++	+++	+++	—	—
	Control 7.	+++	+++	+++	+++	++	—	—
May 14	B 23.	+++	+++	++	+(?)	—	—	—
	B 24.	+++	++	—	—	—	—	—
	Control 5.	+++	+++	+++	+++	+++	—	—
	Control 7.							
May 18	B 23.	+++	+++	++	++	—	—	—
	B 24.	+++	+++	++	+	—	—	—
	Control 5.							
	Control 7.	+++	+++	+++	+++	+++	+++	+

+++ Vigorous phagocytosis. — No phagocytosis.

SUMMARY AND CONCLUSIONS.

In the experiments here detailed rabbits were injected subcutaneously with a mixture of one part benzol (Merck) and two parts olive oil. The doses were, as a rule, so adjusted that each animal received approximately one cubic centimeter of benzol per kilo of body weight.

Sharp individual differences were noted in the effect of the injections upon the leucocytes and the red blood cells in different animals.

A depression in the production of hemolysins, agglutinins, and opsonins was evident in the animals injected with benzol as compared with normal controls injected with the same antigen. The depression was most marked in the case of hemolysins and least so in that of opsonins.

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STUDIES IN EXPERIMENTAL TRANSFUSION.*

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- I. NORMAL ISO-AGGLUTININS IN CATS.
- II. THE DEVELOPMENT OF ISO-HEMOLYSINS AND ISO-AGGLUTININS AS THE RESULT OF TRANSFUSIONS IN CATS.
- III. THE EFFECTS OF AGGLUTINATIVE AND HEMOLYTIC TRANSFUSIONS.
- IV. GLYCOSURIA ACCOMPANYING INTRAVASCULAR HEMOLYSIS.

I.

NORMAL ISO-AGGLUTININS IN CATS.—Preliminary to experimental transfusion in cats we made a short study of the normal occurrence of iso-antibodies in their blood.

Ingebrigtsen¹ first described the occurrence of iso-agglutinins in normal cats. He examined forty cats in groups of ten. Only six cats showed agglutinins in the serum which affected the red cells of other cats. These were scattered in such a way that no definite groupings could be determined, such as occur in man and such as have been shown by Ottenberg and Friedman to occur in some of the lower animals. Of these six agglutinative sera, two agglutinated the red blood cells of one other cat, two those of two others, one those of four others, and one those of seven others.

In general we are able to confirm these findings, but on making repeated examinations of the same cats at intervals of a few weeks we find that the agglutinins are not constant. They vary considerably from time to time although those sera which are strongly agglutinative to the cells of a number of other cats usually remain so over considerable periods (see Charts I. and II.).

Auto-agglutination, that is, agglutination of the animals' suspended red cells by its own serum, was found a number of times. Those strongly agglutinative sera which affect the red cells of a large number of other animals are usually those which are auto-agglutinative.

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This fact together with the occurrence of spontaneous variations in agglutinative power from time to time would seem to rob iso-agglutination as it occurs in cats of some of its biological significance in differentiating individuals, or groups of individuals, of the same species.

Our observations were made on forty cats taken in various groups of ten or twelve at irregular intervals extending over about six months. Independent readings of the results by each of us were made in all cases. The technic followed was that of Epstein and Ottenberg.²

II.

THE DEVELOPMENT OF ISO-HEMOLYSINS AND ISO-AGGLUTININS AS THE RESULT OF TRANSFUSIONS IN CATS. — Iso-hemolysins do not occur among normal cats. The only exception to this, in a great number of tests, was the hemolysis of the cells of one cat in a series of twelve by the serum of a cat (22), which had been used only as a donor for two transfusions. This hemolytic power was absent in tests repeated ten days later (see Charts V. and VI.).

It was thought likely that by repeated injections of blood or by direct transfusions it would be possible to develop iso-hemolysins in cats, as has been done in other animals. The present experiments were undertaken for the purpose of studying the method of their development. It seemed probable from experimental dog transfusions³ and also from the fact that iso-hemolysins in man only develop on the basis of already formed iso-agglutinins, that hemolysins would only develop in animals which already possessed agglutinins for the blood of the donors used. This did not prove to be the case. Hemolysins developed in the serum of some animals that had no agglutinins as well as in animals in which agglutinins were present. They failed to develop in the serum of some animals transfused from donors whose cells were agglutinable.

Twenty-nine transfusions were done on seven cats. The intention was to do repeated transfusions on the same cat, so far as possible from the same donor or from donors showing

similar agglutination reactions. Two of the recipients, however, received only one transfusion each, one received two transfusions, two three transfusions, one eight transfusions, and one ten transfusions. The transfusions were direct from artery to vein or vein to vein by means of the Elsberg or Bernheim canula (usually the latter). They were done at intervals of three to five weeks. Ether anesthesia was used. The donors and recipients were weighed accurately before and after transfusion. The amounts transfused on each occasion varied between thirty and eighty grams, the average being about fifty grams. The symptomatology will be discussed later.

The development of new agglutinins. — A large number of cats which were used as blood donors, or were not used in transfusions at all, and five cats which were repeatedly used as recipients in blood transfusions were retested at intervals during a year with regard to agglutination towards one another's blood. It was found that those cats which were not transfused and whose serum at the beginning agglutinated the red cells of very few or no other cats showed practically no change in agglutinative power. Occasionally, however, they developed agglutinins for the blood of several cats which they had not previously agglutinated. The cats which received repeated transfusions, and which had no or very few agglutinins at the start, developed marked agglutinins for a large number of other cats, usually after two transfusions. When no further transfusions were done these agglutinins generally disappeared after a few months. They could be made to re-appear by again performing transfusions. One cat, which was strongly agglutinative to the majority of other cats before any transfusions were done, remained so after two transfusions had been done, but two months later was found to have lost all agglutinins. The development of iso-agglutinins was apparently independent of whether the cells of the donor cat in each case were agglutinable by the recipient's serum or not.

The development of iso-hemolysins after transfusions. — Of the seven cats which were recipients in transfusions, three developed iso-hemolysins. The serum of the first of these three, Cat 16, was found to be hemolytic for the cells of four out of nine cats one month after the first transfusion. This transfusion was from a donor whose cells before transfusion were slightly agglutinated by the serum of the recipient cat. Cat 16 subsequently escaped and could not be observed further.

The second cat (Cat 19) was transfused from a donor whose cells its serum did not agglutinate, but two months after the second transfusion the serum was found to be strongly hemolytic for the cells of the donor as well as for those of three other cats. The four cats whose cells were susceptible to the serum of Cat 19 were the identical ones whose cells were susceptible to the serum of the preceding Cat 16. Two of the three whose cells were laked were the respective donors of the two cats. It thus appears that there is a definite group or that there are at least two definite groups among cats with regard to artificially developed iso-hemolysins (see Tables III. and IV.). Cat 19 was then transfused ten times over a period of about a year from these cats and from other cats for whose cells its serum was subsequently found to be hemolytic. The serum remained hemolytic toward the same cats throughout the entire time.

The third cat (Cat 18) was transfused five times, at intervals of about a month, from cats whose cells its serum agglutinated. Nevertheless, no hemolysins developed until two months after the sixth transfusion. This sixth transfusion was from a new donor which had never been used before and whose cells were strongly agglutinated by the serum of Cat 18.

The four other cats which were transfused a smaller number of times, one, two and three times, respectively, failed to develop hemolysins. None of the other cats kept under observation ever developed iso-hemolysins although they were repeatedly tested for them.

It thus appears that iso-hemolysins may develop after one

or more transfusions. Probably their appearance or failure to appear depends on the use as donor of a cat belonging to the particular biological group whose cells are susceptible to the hemolysin which the particular cat used as recipient is capable of developing. This would appear to be the case from the failure of Cat 18 to develop hemolysins after five transfusions from four different donors and its development of hemolysins after one transfusion from a fifth donor. Thus it appears probable that the four cats which failed to acquire hemolysins after a smaller number (not more than three) of transfusions would have done so if further trials with new donors had been made.

The titer of the hemolysins developed was not determined, but in mixtures containing equal amounts of fresh serum and of five per cent cell suspension laking occurred in most cases in ten or fifteen minutes at room temperature.

III.

THE EFFECTS OF AGGLUTINATIVE AND HEMOLYTIC TRANSFUSIONS. — In the cases where agglutination alone was found in preliminary tests *in vitro*, there were no symptoms whatever after transfusion and no subsequent changes different from those where no agglutination occurred.

The effect of the transfusion of blood whose cells were susceptible to laking by the serum of the recipient cat was regularly an attack of hemoglobinuria, presumably caused by the destruction of the received blood. This occurred in every one of the ten hemolytic transfusions with the exception of one in which only jaundice developed. Hemoglobinuria was never observed in any of the seventeen non-hemolytic transfusions. This complete correspondence between test-tube hemolysis and intravascular hemolysis confirms the opinion that similar tests for human transfusions can be relied on completely to prevent hemolytic accidents.

The hemoglobinuria was accompanied, as we found in numerous instances, by hemoglobinemia, which occurred within a half hour of the time of transfusion, possibly sooner. Frequently cats did not pass any urine at all for the first

twenty-four hours. The urine contained not only dissolved hemoglobin but sometimes shadows of red cells and numerous blood casts as well. There was regularly a pronounced leucocytosis accompanying the hemoglobinuria (as was also observed in experimental dog transfusions³). On the second and third days the hemoglobin in the urine was replaced by bile and usually the cats were jaundiced.

Blood smears made immediately after transfusion showed on a number of occasions that the hemoglobinemia was accompanied by phagocytosis of red cells in the circulation. This phagocytosis was accomplished to some extent by polymorphonuclear cells, but to a greater extent by large mononuclear cells with more deeply staining cytoplasm than is seen in the large mononuclear cells of human blood.

One entirely unexpected accompaniment of the hemoglobinuria was glycosuria. This led to some further experiments and will be discussed under a separate heading below.

The hemoglobinuria not only led to the destruction of the received blood cells, but had a further effect in producing a pronounced anemia. A gradual destruction of the animal's own blood cells occurred in the days and weeks following the hemoglobinuria. On the other hand, in transfusions where no hemoglobinuria occurred the result was a plethora. Thus in Cat 4 after the second transfusion the red cells were raised from 7,800,000 (an approximately normal count for a cat) to 12,200,000.

In Cat 18, which only developed hemolysins after the sixth transfusion, the red cells were raised as the result of the first six transfusions from 6,700,000 to 12,800,000. Two months after the sixth transfusion (cat in the meantime having developed iso-hemolysins and presumably having destroyed a good deal of the blood which it had received) looked very anemic and smears showed the presence of many nucleated red cells. The seventh transfusion now resulted in jaundice, and the following day the red cells were found to be 7,000,000. A month later they had sunk to 3,600,000 and an eighth transfusion was done which resulted in a marked hemoglobinuria. At the end of the transfusion the red cells

had risen to 8,000,000, but, on the following day, they had sunk again to 6,700,000 and eleven days later to 4,650,000, so that presumably most of the blood cells received were destroyed.

Cat 19 showed a similar course, the red cells being raised from 8,500,000 to 10,800,000 two weeks after the first (non-hemolytic) transfusion. Two months later (the cat in the meanwhile, as the result of the second transfusion, having developed iso-hemolysins) the red cells had sunk to 7,100,000. Three days after the third transfusion, at which time the cat's serum was strongly hemolytic to the donor's cells, and which was accompanied by a pronounced hemoglobinuria, the red cells had fallen to 4,600,000. After a two months' rest the cat's red cells rose again to 7,740,000, and then as a result of three transfusions accompanied by hemoglobinuria the red cells fell again in three months to 3,300,000. Normoblasts and megaloblasts were present, and there was irregularity in the size and staining of the red blood cells, some of them showing pale centers, others showing polychromatophylia.

IV.

GLYCOSURIA ACCOMPANYING INTRAVASCULAR HEMOLYSIS. — In the first hemolytic transfusion on Cat 19 routine examination of the hemoglobinuric urine revealed the fact that sugar was present. After removal of the albumin by boiling with acetic acid, the sugar was determined, using Rudisch's solution, and was found to be one per cent. In six of the seven similar transfusions with the same cat the same phenomenon was observed. In one of them sugar was absent in spite of the fact that hemoglobin was present. In one of the hemolytic transfusions on Cat 18 sugar was also present. These cats showed no glycosuria at other times.

The amount of sugar was always small, usually a fraction of one per cent. It was demonstrated by the reduction of Fehling's and Nylander's solutions and on several occasions by the fermentation test and by the polariscope. In six

transfusions in which hemoglobinuria did not occur no sugar could be demonstrated.

It was thought advisable to ascertain which particular factor in the transfusion caused the glycosuria. Accordingly, immune sera were prepared by the injection of a number of rabbits with either whole defibrinated cat's blood, washed red blood cells, or cat's serum. Of these three sera, those prepared by the injection of whole blood and of washed red cells were found to be powerfully hemolytic to cat's red cells. The one prepared by the injections of serum was feebly hemolytic.

The intravenous injections of the anti-whole blood serum into cats, in amounts varying from .4 cubic centimeter up to two cubic centimeters, caused a pronounced hemoglobinuria in four of seven cats and a glycosuria likewise in four of the seven. One cat had glycosuria without hemoglobinuria and one hemoglobinuria without glycosuria. In the first of these experiments the presence of sugar was determined not only by reduction and fermentation tests and by the polariscope, but also by the formation of crystals of phenylglucosazone. The serum which had been prepared by the injection of washed red cells (known as anti-red blood cell serum) injected intravenously into cats in amounts of from one to two cubic centimeters also produced a marked hemoglobinuria, but no glycosuria on either of the two occasions in which it was used. Both of these hemolytic sera (the anti-whole blood and the anti-red cell) were highly toxic, and amounts over 2.5 cubic centimeters usually killed the cat within a few minutes. Blood removed at once from the portal or pulmonary veins of these cats showed macroscopic red-cell agglutination, and at room temperature hemolysis occurred in fifteen to twenty minutes. On the other hand, the anti-serum serum was not toxic in amounts up to three cubic centimeters. One cat which was injected intravenously with anti-red blood cell serum had a marked hemoglobinuria the next day and showed at autopsy, one month later, thrombosis of the pulmonary arteries supplying both lower lobes and large pulmonary infarcts in these lobes. The serum prepared by injecting rabbits

with cat's serum (anti-serum serum) produced no hemoglobinuria in any of four experiments, but after two of the injections did produce a slight glycosuria (.4 per cent of sugar in one experiment and a trace in the other). The injection of distilled water intravenously into cats (ninety and one hundred and twenty cubic centimeters in two experiments) produced a pronounced hemoglobinuria but without sugar.

With this small number of experiments we are unable to determine the significance of the glycosuria which sometimes accompanies hemoglobinuria. There is no doubt about the substance present being sugar, although in several of the experiments in which there was no sugar there was present in the urine some substance which produced distinct reduction (yellow color) of Fehling's solution without, however, the formation of a precipitate.

The failure of sugar to appear in the distilled water experiments and in the anti-red-cell serum experiments would suggest that its occurrence is more probably connected with antibodies directed against some serum constituent than with hemolysis. The subject requires further study.

CONCLUSIONS.

I. Iso-agglutinins in cats' blood do not form distinct groups. They are relatively feeble antibodies, and vary considerably from time to time in the same cats.

II. Iso-hemolysins are seldom, if ever, found among normal cats, but they often appear in the recipients of blood transfusions. Apparently their occurrence or failure to occur depends on some biological property of the blood of the donor cat. The iso-hemolysins which appear are selective, affecting not only the blood of the donor cat but of certain others also. Therefore it seems that there are biological groups within the species in this case, just as there are among goats (Erich's fundamental experiments), among human beings (Moss), and, as Von Dungern has shown, among cats.

III. The direct transfusion of iso-agglutinative blood has no immediate harmful effects.

IV. The transfusion of blood whose cells are susceptible to laking by the recipient's serum produces a marked hemoglobinemia and hemoglobinuria, with intravascular phagocytosis of red cells, a reactive leucocytosis, and usually glycosuria.

V. This intravascular destruction of another animal's blood exerts some toxic effect, so that the recipient animal itself develops an anemia with the appearance of bone marrow cell forms in the circulating blood. There is a tendency to spontaneous recovery from this anemia.

VI. Glycosuria sometimes accompanies the intravascular destruction of blood. It does not seem to be dependent on hemolysis alone, but on some other factor, not as yet understood.

TABLE I. (Feb. 1, 1913.)

Normal iso-agglutinins.

Readings after one-half hour at room temperature.

	2	3	4	5	6	7	8	11	12	13	14	15	16	17	18	19
2	—	—	—	—	—	—	—	—	—	+	—	—	+	+	—	—
3	—	—	—	—	—	—	—	—	—	+	—	—	?	—	—	—
4	—	—	—	—	—	—	—	—	—	+	—	—	+	+	—	—
5	—	—	—	—	—	—	—	—	—	+	—	—	?	—	—	—
6	—	—	—	—	—	—	—	—	—	+	—	—	?	+	—	—
7	—	?	—	—	—	—	—	—	—	+	—	—	+	+	—	—
8	—	?	—	—	—	—	—	—	—	+	—	—	?	+	—	—
11	—	?	—	—	—	—	—	—	—	+	—	—	+	+	—	—
12	—	—	—	—	—	—	—	—	—	+	—	—	+	+	—	+
13	—	—	—	—	—	—	—	—	—	+	—	—	+	+	—	?
14	—	+	—	—	—	?	—	—	—	+	—	—	?	—	—	—
15	—	?	—	—	—	+	—	—	—	+	—	—	—	?	—	—
16	—	+	—	?	—	?	—	—	—	+	—	—	?	+	—	—
17	—	+	—	—	—	—	—	—	—	+	—	—	?	—	—	—
18	—	—	—	—	—	—	—	—	—	+	—	—	?	—	—	—
19	—	?	—	—	—	—	—	—	—	?	—	—	—	?	—	—

In the tables, + indicates agglutination; — indicates absence of agglutination; ? indicates slight or questionable agglutination; H indicates hemolysis.

The vertical columns indicate sera, the horizontal lines red cells of the cats whose numbers are given.

TABLE II. (Feb. 5, 1913.)

Normal iso-agglutinins.

Readings after two hours at room temperature.

	3	4	5	6	7	8	11	12	13	14	15	16	17	18	19
3	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—
4	—	—	—	—	—	—	—	—	+	—	—	+	+	—	—
5	—	—	—	—	—	—	—	—	+	—	—	+	+	—	—
6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
7	—	—	—	—	—	—	—	—	+	—	—	+	+	—	—
8	—	—	—	—	—	—	—	—	—	—	—	+	+	—	—
11	—	—	—	—	—	—	—	—	+	—	—	+	+	—	—
12	—	—	—	+	+	+	—	—	+	—	—	+	+	—	+
13	—	—	—	—	—	—	—	—	+	—	—	+	+	—	—
14	—	—	—	—	—	—	—	—	+	—	—	+	+	—	—
15	—	—	—	—	—	—	—	—	+	—	—	+	+	+	+
16	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—
17	—	—	—	—	—	—	—	—	—	—	—	+	+	—	—
18	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—
19	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—

Tables I. and II. show the variability of the iso-agglutinins. Cats which have agglutinins on one occasion for the red blood cells of many other cats, possess agglutinins on another occasion for the red blood cells of about the same number of other cats, but not always the same ones.

TABLE III. (May 16, 1913.)

Agglutinins after transfusions.

Readings after two and one-half hours at room temperature.

	4	6	7	8	11	15	16	17	19
4	—	—	—	—	—	—	+	+	+
6	—	—	—	—	—	—	—	+	+
7	—	—	—	—	—	—	+	+	+
8	—	—	—	—	—	—	+	+	—
11	—	—	—	—	—	+	+	+	+
15	—	—	+	+	—	—	—	?	+
16	—	—	—	—	—	—	+	—	—
17	—	—	—	—	—	—	+	+	+
19	—	—	—	—	—	+	+	—	+

(See footnote, Table IV.)

TABLE IV. (May 16, 1913.)

*Hemolysins after transfusions. Tests for hemolysis.*Readings after two hours at 37 $\frac{1}{2}$ ° C. and twelve hours in ice-box.

	4	6	7	8	11	15	16	17	19
4	—	—	—	—	—	—	—	—	—
6	—	—	—	—	—	—	H	—	H
7	—	—	—	—	—	—	H	—	H
8	—	—	—	—	—	—	—	—	—
11	—	—	—	—	—	—	H	—	H
15	—	—	—	—	—	—	H	—	H
16	—	—	—	—	—	—	—	—	—
17	—	—	—	—	—	—	—	—	—
19	—	—	—	—	—	—	—	—	—

Cat 19 has had two transfusions from Cat 7. Cat 16 has had one transfusion from Cat 11.

Tables show marked development of agglutinins and hemolysins in Cat 19 and in Cat 16. Both cats, though transfused from different donors, have developed hemolysins for the red blood cells of one another's donors and for the red blood cells of two other cats.

TABLE V. (July 5, 1913.)

Further development of hemolysins.

Readings after three hours in thermostat and overnight in ice-box.

	4	6	8	11	15	18	19	20	21	22	23	24
4.....	—	—	—	—	—	—	—	—	—	—	?	—
6.....	—	—	—	—	—	+	H	—	—	+	+	—
8.....	—	—	—	—	—	—	+	—	—	+	—	—
11.....	—	—	—	—	—	—	H	—	—	—	—	—
15.....	—	—	—	—	—	—	H	+	—	+H	—	—
18.....	—	—	—	—	—	—	—	—	—	—	—	—
19.....	—	—	—	—	—	—	—	—	—	—	—	—
20.....	—	—	—	—	—	—	—	—	—	—	—	—
21.....	—	—	—	—	—	—	H	—	—	—	—	—
22.....	—	—	—	—	—	—	—	—	—	—	—	—
23.....	—	—	—	—	—	—	—	—	—	—	—	—
24.....	—	—	—	—	—	—	—	—	—	—	—	—

TABLE VI. (Sept. 16, 1913.)

Development of agglutinins and hemolysins.

Readings after two hours at room temperature.

	4	6	8	11	18	19	22	23	25
4.....	—	—	—	—	+	+	+	—	—
6.....	+	—	—	—	+	H	—	—	—
8.....	+	—	—	—	+	+	+	—	—
11.....	—	—	—	—	+	+H	—	—	—
18.....	+	—	—	—	+	+	+	—	—
19.....	+	—	+	—	—	+	+	—	—
22.....	—	—	—	—	+	+	—	—	—
23.....	—	+	+	—	+	+	+	—	—
25.....	—	—	—	—	+	+	—	—	—

Cat 18, two weeks since last of three transfusions, shows numerous agglutinins.

TABLE VII. (Oct. 18, 1913.)
Variability of agglutinins after transfusions.
 Readings after two hours at room temperature.

	4	6	8	11	18	19	22	23	25	26	27
4.....	—	—	—	—	—	+	—	—	—	—	—
6.....	—	—	—	—	—	H	—	—	—	—	—
8.....	—	—	—	+	—	+	—	—	—	—	—
11.....	+	—	—	—	—	H	—	—	—	—	—
18.....	—	—	—	—	—	+	—	—	—	—	—
19.....	—	—	—	—	+	+	—	—	—	—	—
22.....	+	+	—	—	—	+	—	—	—	—	—
23.....	—	—	—	—	—	—	—	—	—	—	—
25.....	—	—	—	—	—	+	—	—	—	—	—
26.....	—	—	—	—	—	+	—	—	—	—	—
27.....	—	—	—	—	—	+	—	—	—	—	—

Cat 18, one month since last transfusion, shows agglutination of red blood cells of only one cat.

TABLE VIII. (Feb. 3, 1914.)

Further development of agglutinins and hemolysins.

Readings after two hours at room temperature.

	4	8	18	19	23	25	28	29	30	31
4.....	—	—	+	+	—	—	+	+	—	—
8.....	—	—	+	+	—	—	+	+	—	—
18.....	—	—	+	+	—	—	+	+	+	—
19..	—	—	+	+	—	—	+	—	—	—
23.....	—	—	+	H	—	—	+	+	—	—
25.....	—	—	—	+	—	—	+	—	—	—
28.....	—	—	+	+	—	—	+	+	—	—
29... ..	—	—	+	+	—	—	+	—	—	—
30.....	—	—	+	+H	—	—	+	—	—	—
31.....	—	—	+	+	—	—	+	—	—	—

Cat 18 has received three transfusions since last tests recorded in Table VII., and shows again numerous agglutinins.

Cat 19 has developed hemolysins for the red blood cells of two new cats (23 and 30).

The cats for whose red blood cells Cat 19 had previously possessed hemolysins are dead.

TABLE IX. (March 10, 1914.)

Development of hemolysins in two cats.

Readings after two hours at room temperature.

	8	18	19	23	25	28	29	30	31
8	—	—	+	—	—	+	+	+	—
18	—	+	+	—	—	+	+	+	—
19	—	+	—	—	—	+	—	+	+
23	—	+H	H	—	—	+	+	+	+
25	—	+	+	—	—	—	—	+	+
28	+	+	+	—	—	—	—	+	+
29	—	+	+	—	—	+	—	+	+
30	—	+H	H	—	—	+	—	+	+
31	—	+	+	—	—	+	—	+	—

Cat 18 received its sixth transfusion Jan. 23, 1914, from Cat 27, which was used as donor for the first time, and for the red blood cells of which Cat 18 possessed agglutinins. Cat 27 died Jan. 27, 1914, which prevented further tests with its blood.

The above table shows that Cat 18 has developed hemolysins for the red blood cells of Cats 23 and 30.

TABLE X. (July 23, 1914.)

Loss of agglutinins and hemolysins after discontinuance of transfusions.

Readings after two hours at room temperature.

	8	18	23	25	28	30	32	33	35	36
8.....	—	—	—	—	+	—	—	—	—	—
18.....	—	—	+	—	+	—	—	+	—	—
23.....	—	+	—	—	+	—	—	—	—	—
25.....	—	—	—	—	+	—	—	+	—	—
28.....	—	—	—	—	+	—	—	+	—	—
30.....	—	+	—	—	+	—	—	+	—	—
32.....	—	—	—	—	+	—	—	+	—	—
33.....	—	—	—	—	+	—	—	—	—	—
35.....	—	—	—	—	—	—	—	+	—	—
36.....	—	—	—	—	+	—	—	—	—	—

Cat 18 was the recipient of its seventh and eighth transfusions March 11 and April 10, 1914, Cat 23 being the donor. Cat 18 developed hemoglobinuria after the eighth transfusion, and possessed hemolysins for the red blood cells of Cat 23 on April 10, 1914 (not in tables).

The above table shows that Cat 18, three and a half months after its last transfusion, has lost most of its agglutinins and hemolysins. The only agglutinins which it has retained are for the red blood cells of Cats 23 and 30, for which it previously also possessed hemolysins.

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A BACTERIOLOGICAL STUDY OF AN EPIDEMIC OF SEPTIC SORE THROAT.*

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The epidemic we have to report is of especial interest because of the complete and conclusive results obtained. The epidemiological facts are briefly as follows:

The outbreak occurred during June, 1914, in Rockville Centre, Long Island, a village of 4,250 inhabitants. It was soon evident that the common element among the infected persons was their milk supply. The suspected milk came from one dairy (Oceanside) housing twenty-two cows. This dairy also obtained some milk from two other small farms (R. and C.), and distributed about four hundred quarts a day to about one hundred and seventy-three customers. On investigation it was found that two hundred and five out of two hundred and thirty-two cases obtained their milk from this source. Other milkmen delivered about twelve hundred quarts a day. That is, a milk supply making up twenty-five per cent of the total had at least ninety per cent of the cases.

All three farms were investigated. At the Oceanside Dairy it was learned that Miss W., the daughter of the owner, developed a sore throat on April 16, 1914. On May 9th, Mrs. W., the owner, developed sore throat, but claimed that she had remained away from the dairy until May 28th. On May 11th the driver developed sore throat and on June 9th the milker. The major part of the milking was done by Mrs. W. and the milker, but the driver helped at times and possibly others. All apparently helped as needed with the work.

Isolated cases of sore throat developed in users of this milk on April 21st, 27th; May 17th and 21st. Two cases occurred in a family using milk from their own cow on May

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30th. No information is available of other cases at this time, but we are inclined to believe that these as well as those on the farm were sporadic and contact cases.

From June 1st to 5th the number increased, the main part of the outbreak occurring between June 6th and 14th. A few cases a day occurred until June 30th and two isolated ones on June 29th and 30th.

The material for bacteriological investigation consisted of moist swabs from the throats of each individual on the three farms and from a number of cases, as well as a culture from a complicating peritonitis. Milk was collected separately from each quarter of the udder from all the cows.

The samples of milk on being received were placed on ice over night and smears made from the sediment. The samples from five cows showed a moderate number of streptococci in one or more quarters. One of these cows showed evidences of mastitis on physical examination. Another cow (No. 21) showed a moderate number of streptococci in samples from two quarters and an enormous number in the sample from another quarter. The milk from this quarter was flocculent, due apparently to partial coagulation of the casein and separation of the whey. All of the positive samples were inoculated on blood-agar plates and several pure cultures isolated and their characteristics determined. The samples were obtained June 15, 16, 1914.

CULTURAL CHARACTERISTICS OF STREPTOCOCCI FROM COW'S MILK.

Cultures from —	Blood Plates.	Action on Washed Blood Cells.	Fermentation of* —		
			Raffinose.	Mannite.	Salicin.
Cows 2, 3, 4, 5, and 6 . .	No hemolysis.	No hemolysis.	—	—	+
Cow 21 (3 quarters) . .	Hemolysis.	Hemolysis.	—	—	+

* None fermented inulin.

The moist swabs from the throats of those on the farms and from the cases were received at the laboratory within

about two hours after collection. The material was then streaked on the surface of blood agar plates. The varying percentage of hemolyzing streptococci present is striking. For this reason their percentage is given with the type of case.

Cases. — Obtained June 15 and 16, 1914 :

Adult — convalescent	Hemolyzing types,	50%
Child, 2 years, slight throat symptoms, marked cervical node involvement	“ “	10%
Convalescent, 15 years old	“ “	100%
Adult, sick one week, relapse, marked backache and depression	“ “	48%
Adult, sore throat for 36 hours, no prostration ...	“ “	20%
Adult, sore throat for three days	“ “	40%
Adult, sore throat, one week	“ “	85%
Child, subacute stage, lymph nodes enlarged	“ “	10%
Child, 20 months, ill eight days, temp. 105° F. ...	“ “	20%
Child, 6 years, convalescent	“ “	20%
Child, 3 years, throat inflamed, cervical nodes enlarged	“ “	2%
Adult, acute sore throat	“ “	5%

Oceanside Dairy. — Obtained June 15 and 16, 1914 :

Mrs. W. — Sore throat, May 9th	Hemolyzing types,	0%
Driver. — “ “ “ 11th	“ “	0 5%
Milker. — “ “ June 9th	“ “	20.0%
Washerwoman. — Illness denied, enlarged tonsils,	“ “	0.5%

Farm R. — Obtained June 15 and 16, 1914 :

Mrs. E. R. (22). — No history of illness, enlarged tonsils	Hemolyzing types,	30%
M. R. — No history of illness	“ “	0
N. J. — “ “ “ “	“ “	0

Farm C. — Obtained June 15 and 16, 1914 :

Mrs. C. — No history of illness	Hemolyzing types,	0
H. C. — “ “ “ “	“ “	0
M. C. — “ “ “ “	“ “	0

Pure cultures were isolated from each case and included, not only several hemolyzing types, but also several of the non-hemolyzing types. The cultural characteristics were then determined.

On June 29th material was received from a case (Mrs. B.) which developed peritonitis as a complication. This culture was extremely important. Being of the invasive type it was presumptively that producing the sore throat.

The non-hemolyzing types were studied only in relation to the similar ones isolated from five of the cows. Most of the strains could be excluded because of their ability to ferment inulin or raffinose, to produce methhemoglobin or inability to ferment salicin. Very few strains had the fermentative characteristics of the bovine strains and nearly all which did came from the throats of the non-exposed on Farms R. and C. Because of these findings the non-hemolyzing types can be excluded.

The hemolyzing types obtained from the throats of the cases and of the persons on the Oceanside Dairy and the one culture from a complicating peritonitis were not only the same in their cultural characteristics, but were similar to the hemolyzing strains isolated from the one cow. (See Table.)

Only one hemolyzing type was isolated among the non-exposed (Mrs. E. R. (22) Farm R.). This strain although it gave the same sugar fermentations was different in many of its characteristics. Its growth in broth was very abundant, forming a thick soft flocculent sediment, which showed a faint orange pigmentation. It fermented salicin with great rapidity and gave positive fermentation results with unfavorable media when all the other strains remained negative. Morphologically, it was sharply different from the other strains.

Rabbits were immunized with the peritonitis strain (Mrs. B.) and the serum used for agglutination. Although the serum was only of moderate titer, the results are very sharp. (See Table.)

A few streptococci from other sources were studied for comparison, including several strains from Dr. Theobald Smith, as well as cultures obtained from a widespread outbreak of sore throat in Westchester County, N.Y. (See Table.)

This outbreak of septic sore throat occurred in Westchester County during March, 1915. The investigations of Prof. C. E. A. Winslow¹ led him to believe that this outbreak was partly milk-borne and partly due to contact infection. Throat swabs were obtained from ten cases² and plated on

blood agar. A variable percentage of slightly hemolyzing colonies were present. Because of the slight hemolysis the later material was used for poured blood plates as well. Compared with the streaked plates, the poured plates showed an apparent higher percentage of hemolyzing types and more important, typical wide zones of hemolysis. Although the streaked plates are more convenient, it is evident from this that they may give confusing results.

The cultures isolated were indistinguishable culturally from those isolated in the Rockville Centre outbreak, but were different agglutinatively. (See Table.)

The bacteriological findings in the Rockville Centre epidemic, that is, the isolation of a distinct race of streptococci from the throats of the cases and from a complicating peritonitis as well as from the throats of the persons and from a cow on one farm, and the absence of this streptococcus in the throats of persons on the other farms, these facts added to the epidemiological data, that is, the presence of sore throat on the one farm before the general outbreak, give an almost complete certainty that the following sequence of events took place.

Miss W., April 16th. Sore throat; contact infection to Mrs. W., May 9th; contact infection from one of these to driver, May 11th; one of the latter two, probably Mrs. W., infected the cow; multiplication of the streptococci in the milk ducts and in the milk itself with contamination of the mixed milk, culminating in the outbreak.

The facts, therefore, give strong added evidence to the view that infection in milk-borne sore throat is of human and not of bovine origin. The fact that the cow infected with the "human" streptococci had no physical evidences of mastitis, whereas another cow having mastitis yielded another unrelated "bovine" variety of streptococcus, is also of value as evidence in this connection. Previous observations have shown that some types of human streptococci can multiply

for a shorter or longer time in the milk of the udder without producing evident mastitis.

It would be needless repetition to present the existing evidence that the streptococcus of milk-borne septic sore throat is of human origin and not due to the streptococci associated with udder inflammation, some strains of which may accidentally possess a high virulence for man. Smith and Brown³ have given a complete analysis of the evidence furnished by their own investigations and by others.

The streptococci isolated in the various epidemics including our own have all been the same culturally with one exception, viz., "Outbreak A" of Smith and Brown. This streptococcus fermented mannite as well as salicin, but did not ferment lactose. It differed also agglutinatively from the streptococci of the Baltimore and Chicago outbreaks. Our own results show, however, that streptococci similar culturally, but from different outbreaks, are not necessarily identical agglutinatively.

Smith and Brown also show that the isolation from the suspected milk of a streptococcus culturally similar to that obtained from cases of sore throat is insufficient to establish the causal relationship. They isolated hemolyzing streptococci, culturally similar, from two cows. One produced quantitatively more acid from dextrose, lactose, saccharose, and maltose. The strain from the other cow agreed quantitatively and in virulence with the human strains. These quantitative differences were associated with differences in agglutinations.

A few points of practical value were noted in our study. One is that relatively few hemolyzing types may be found even where the swabs are inoculated directly upon blood agar. It is evident that they might easily be lost if mixed cultures on Loeffler's blood serum were submitted for examination. Typical hemolysis may not be present on surface streaked blood plates, although it may develop after longer incubation. Serum water is unsuited for the determination of sugar fermentation. All of the type strains failed to ferment salicin using this medium, although prompt fermentation

took place using serum broth or serum water containing one per cent of peptone.

SUMMARY.

Streptococci similar culturally and identical in their agglutination were isolated from cases of septic sore throat and from the udder of one cow, showing no evidence of mastitis, except the peculiar character of the milk from one quarter. The bacteriological as well as the epidemiological facts almost certainly show that the infection was primarily of human origin. In tracing the source of such an epidemic, the effort should be towards finding cases of sore throat among those engaged in producing the milk, not mastitis in the cow alone. If human streptococci are found in mastitis, they are most likely secondary agents in an already existing inflammation due to bovine strains. The streptococci in different epidemics differ culturally and those similar culturally differ in their immunity reactions. Cultural similarity of strains from man and cattle is insufficient to prove their identity. Cultural identity in every detail or immunological identity is essential.

[We are indebted to Dr. Herman M. Biggs, Commissioner of Health, State Department of Health, N.Y., for the opportunity of studying this epidemic, and to Dr. Frank Overton, County Supervisor; Dr. A. D. Jacques, Health Officer of Rockville Centre; Dr. H. D. Gill, Veterinarian of the Department of Agriculture, as well as Dr. M. C. Schroeder and Mr. Russell Sturgis of the New York City Health Department, who coöperated in the epidemiological investigation and supplied us with much of the material for laboratory study. A report of the general results of the investigation was given in "The Monthly Bulletin," New York State Department of Health, July, 1914.]

TABLE. — HEMOLYTIC STREPTOCOCCI.

	Strains.	Fermentation of —			Agglutination, Serum Strains Mrs. B.			
		Raffinose.	Mannite.	Salicin.	1-10.	1-50.	1-100.	Control.
Rockville Centre.	Sore throat	—	—	+	++	++	+	—
	“ “	—	—	+	++	++	+	—
	“ “	—	—	+	++	++	+	—
	Mrs. B., peritonitis .	—	—	+	++	++	+	—
	Cow 21, a	—	—	+	++	++	+	—
	“ 21, b	—	—	+	++	++	++	—
	Mrs. E. R. (22) . . .	—	—	+	+	—	—	—
West-chesler.	Sore throat	—	—	+	+	—	—	—
	“ “	—	—	+	++	±	±	±
Miscellaneous.	Spinal fluid	—	—	+	++	±	±	±
	Mastoid abscess . .	—	—	+	+	±	±	±
	Peritonitis	—	+	+	+	±	±	±
	Otitis media	+	—	+	+	—	—	—
Smith.	Outbreak A	—	+	+	+	—	—	—
	“ B	—	—	+	+	—	—	—

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A METHOD OF PRODUCING TETANUS TOXIN.*

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The present European war has been the occasion for an increased production of tetanus antitoxin in this country. It therefore seems appropriate to place on record the method of making tetanus toxin which has been used with minor modification at the Hygienic Laboratory of the U.S. Public Health Service since 1905, and, at the suggestion of the Laboratory, in most of the establishments manufacturing antitoxin subsequently. This method has proven simple and, above all, has in numerous instances solved the problem of obtaining constantly and at will a toxin of uniformly good potency. At the Hygienic Laboratory the toxin is prepared for the purpose of standardization of tetanus antitoxin in accordance with the American method,¹ while in the antitoxin establishments it is prepared for the active immunization of horses.

In every instance within our knowledge this method of making tetanus toxin in bulk — fifty to one hundred liters at a time — has given good results, without a failure; and at the Hygienic Laboratory the strength of the toxin produced has been nearly uniform over a number of years.

The method is as follows:

The stock strains are carried on glucose-agar slabs eight centimeters deep at 15° C. (after a short preliminary incubation at 37° C.); they are transplanted every four to six months, the growth reaching usually to within one millimeter of the surface. The cultivation just prior to making a toxin is in tubes of freshly prepared one per cent glucose broth twelve centimeters deep. This gives a good growth in forty-eight hours in the first tube, in twenty-four hours in the second and third transplants, and in sixteen hours on the fourth. Daily transplants, with daily microscopical examinations for

* Received for publication Aug. 30, 1915.

purity, are then made for one to three weeks, when the flasks are inoculated. For one hundred liters of medium, fifty kilograms of round steak are ground up, infused twenty-four hours in an ice-box with one hundred liters of water, strained through cheese-cloth and squeezed out in a tincture press till the original amount of liquid is yielded. This is heated in streaming steam one hour, filtered through paper, titrated against twentieth normal sodium hydroxide while boiling, normal sodium hydroxide added to neutralize, and .5 per cent sodium chloride and one per cent peptone put in. The broth is heated again one hour in streaming steam and the reaction again adjusted to neutrality to phenolphthalein; the broth is then filtered through paper into liter Erlenmeyer flasks, one thousand cubic centimeters being placed in each, which are then steamed without pressure for one and one-half hours. If not needed at once, these flasks may be stored for a period of two weeks or less. Just before planting a one per cent solution of powdered glucose C.P. is added and the medium again heated one and one-half hours in streaming steam, cooled to 40° C., and immediately inoculated, one cubic centimeter of a twenty-four-hour culture being placed a few centimeters below the surface. No oil or other method is used to secure anaërobiasis. Good growth is obtained in sixteen hours, but incubation is allowed to go on undisturbed for fifteen days at 37° C., when the flasks are examined for purity of growth, and the toxin filtered through Berkefeld candles by vacuum.

There seems to be little choice among the strains used, for the variation in toxicity between different flasks inoculated with the same strain is in general as great as that between flasks containing different strains.

The important points in this method appear to be the method of preparation, freshness, and reaction of the medium, the preliminary cultivation, and the growth without the use of oil, vacuum, inert gas, or pyrogallate — a great advantage; the anaërobiasis is attained by inoculating liberally into liter flasks immediately after steaming.²

The successful use of this method effectively disproves the statement often repeated in text-books that the so-called

"aërobic" cultivation of the tetanus bacillus is attended with a loss of its toxin production. It is possible that meat infusion, even when passed through a Schleicher and Schuell filter No. 588, retains some of the characteristics of the organ broth of Smith^{3, 4} (see also Tarozzi,⁵ Wrzosek,^{6, 7} and Harrass⁸).

Liefmann,⁹ it is true, was able to grow anaërobes without exclusion of air in meat infusion if decanted, clear, even after boiling, but not if heated with the meat before infusing, and not at all in filtrates. Our flasks of tetanus must in the course of a few days have a considerable oxygen content in the depths due to the "streaming" action of evaporation and concentration at the surface, as explained by Phelps,¹⁰ and even more by reason of the minute bubbles of gas produced by the bacilli and causing an agitation of the bouillon as they rise to the surface. The growth, in fact, appears throughout all layers of the flasks and tubes, but at the end of incubation tends to accumulate toward the bottom.

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FURTHER INVESTIGATIONS INTO THE ETIOLOGY OF THE
PROTOZOAN DISEASE OF TURKEYS KNOWN AS BLACK-
HEAD, ENTERO-HEPATITIS, TYPHLITIS, ETC.*

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In 1895 the writer¹ published an account of the pathological anatomy and parasitology of a highly fatal infectious disease of turkeys commonly known as blackhead. Since then but few opportunities have presented themselves to enable him to supplement the early work with experiments designed to determine accurately the life cycle of the parasite. Such a determination would solve certain practical questions concerning the mode or modes of infection and their prevention. For some years the writer received diseased turkeys from Dr. Cooper Curtice, who was carrying on experiments at the Rhode Island Experiment Station. A study of this material, however, did not add anything to what was known. During these years Dr. Curtice^{2,3} himself was struggling with the problem of transmission and his experiments though not conclusive are pioneers in field work with this disease.

In 1910 the attempt of Cole and Hadley⁴ to show that blackhead was nothing more than a coccidiosis induced the writer to take up the subject again and the few experiments that were possible with the means at hand up to the present are included in the present paper, which may be considered merely a progress report bringing the work in Harvard University to a conclusion. This work will be continued until the problems discussed in this paper shall have been subjected to the test of satisfactorily controlled field experiments.

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PATHOLOGY AND PARASITOLOGY.— Since the few studies relating to this disease have appeared in Government publications not easily accessible, and since the writer's original paper has been out of print for a number of years, it has seemed advisable to briefly summarize our present information before detailing the additional experiments. Leaving at present out of consideration the symptoms of blackhead which are not specially characteristic, we will limit ourselves to a very brief statement of the gross and microscopic appearances of the lesions and of the characters of the micro-organism associated with them.

The lesions in blackhead are almost entirely limited to the ceca and the liver. In rare cases the liver is free from visible changes. This occasional absence of liver lesions led the writer in the early report¹ to regard the exclusive affection of one or both ceca as a bacterial disease, differing from the entero-hepatitis of blackhead. This view is now considered untenable, and it is highly doubtful that any other cecal affection of turkeys exists which closely resembles that of blackhead. The absence of liver disease is accounted for by the simple hypothesis that the resistance of the affected animal in some way keeps the parasites passing from the blood vessels of the ceca into the liver from multiplying.

Besides the involvement of ceca, mesenteries of ceca, and liver, the writer has recently seen the extension of the disease by contiguity in one case from a liver focus to the wall of the adjacent proventriculus. In another case the process extended from a focus of one cecum into the wall of the contiguous small intestine. In a third case the disease had invaded primarily the large intestine (between openings into ceca and cloaca) and extended thence into a part of the subjacent kidney. These extensions are very rare and deserve the name of pathological curiosities. Other lesions, such as false membranes on liver and on the walls of air-sacs are due to secondary bacterial invasions not infrequent in the long-standing cases of more resistant older birds.

The most striking abnormal condition of one or both

ceca is the filling up of the lumen with a tough, leathery, yellowish mould usually made up of concentric layers and centrally hollow. In many instances this is attached to the mucosa and when forcibly pulled away leaves an irregular, roughened denuded surface. Not infrequently the mould lies free and is covered with intestinal contents. It represents one or more outpourings of coagulable material from the mucosa. From this extreme involvement all gradations may be found down to a very small isolated necrotic patch or patches, or, in late cases, mere scars of the membrane.

The wall of the cecum containing the mould of exudate may be but slightly thickened or it may be several millimeters thick, either uniformly or in patches. The wall then shows from the serous aspect patches of yellowish discoloration. The thickening may creep along and involve the entire mesentery attaching the cecum to the small intestine. In such cases the wall of the latter has been found free from disease, however (Plate XII., Fig. 2).

The liver is involved in perhaps all fatal cases. Among fifty-eight consecutive cases, recently examined, the liver was free from macroscopic evidences of disease in seven, or in about twelve per cent. The lesion takes the form of spherical foci, up to one centimeter or even more in diameter. These foci when situated at the surface are seen as circular spots differing in appearance according to the age of the lesion and virulence of the infection. The earliest stages are very faintly outlined as circular slightly depressed areas of a mottled dark red. In the later stages these spots become yellowish. At first the yellowish material is sprinkled in the area in minute particles, giving the area a wheel-shaped, filigree-like appearance. In other and later stages the focus may be uniformly yellowish, of the consistence of firm cheese. Under the fingers it is easily mapped out as a hard mass from the softer enveloping liver tissue and is crushed with difficulty between slides. In still later stages (of repair) these spots have a mottled grayish-yellow appearance and are less sharply outlined.

In the bird which has died or which has been killed

blackhead is readily diagnosed. When the disease is not at once evident from an inspection of the ceca and the liver, the former should be slit open and after the contents have been gently removed by washing, the mucous membrane should be searched for isolated areas of attached exudation, local thickenings of the wall, roughened discolored areas, or (in recovered cases) for scars. The liver should be examined on all its surfaces and by means of sections for foci like those described. Among the nearly two hundred cases autopsied, the writer has seen the exceptional invasion of contiguous organs, as stated above, in only three.

In the ceca of turkeys are small circular elevations one to two millimeters at the base with a central crater-like depression. These are pits of the mucosa into which the epithelium dips rather deeply and thereby forms branching tubules which are embedded in lymphoid tissue (Plate XII., Fig. 1; Plate XIII., Figs. 3 and 4). Attention was focused upon these for a time, since in some cases the necrosis and exudation seemed to start from them. No definite information was obtained on this point. The wide extent of the disease involving the entire mucosa has led me to the view that this lymphoid tissue is not a necessary or even a frequent starting point of the lesion.

The significance of the fact that in some instances only one cecum is affected is not clear. Two hypotheses may be invoked. The infection may be so diluted that it reaches only one cecum. Or, the virus may require some preliminary help in the form of some injury to the mucous membrane. The absence of coccidia and higher animal parasites, such as round worms, from most of the cases examined since 1910 indicates that injuries do not play any significant part in preparing the way for the ameba. It is not improbable that quantity of infection plays the essential part, for the occurrence of double and single infection varies much with groups of cases. Thus in one group of eighteen cases eight, in another group of twenty-seven eighteen, had but one cecum involved. In a third group of thirteen only three had a

single cecum diseased. Taking the three groups of fifty-eight cases together we have twenty-nine, or just fifty per cent, with but one cecum affected.

The disease attacks both young and adult turkeys. It begins its ravages soon after the young leave the shell, for I have seen advanced, fatal cases in birds not more than three weeks old. Large numbers die within the two first months. Of those exposed which survive, many have had lesions in the ceca which have healed as shown by scars of the mucous membrane or by remains of necrotic masses still attached to the underlying tissues. The writer has seen active disease in birds over a year old.

In the early report, the writer found protozoan parasites which in the fresh condition appeared as "round homogeneous bodies with a sharply defined, single-contour outline. . . . Within these bodies and situated somewhat eccentrically is a group of very minute granules, probably representing a nuclear structure." They were found to vary from 8 to 15 μ in diameter. In tissues fixed and stained they ranged from 6 to 10 μ . "In most of these bodies a minute, distinctly blue ring is seen situated centrally or somewhat eccentrically and representing the nuclear membrane. This is about 2 μ in diameter. With high powers, a very minute (nucleolar?) point may be seen in some parasites within the nucleus."

The parasite was described as situated within the connective tissue and lymph spaces of the mucosa and submucosa. Giant cells were frequently encountered containing them. Owing to the characters of the parasite and the general analogy of this disease with amebic dysentery in man, the parasite was tentatively regarded as an ameba and named *A. meleagridis*. Inasmuch as a certain definite type of mobility has always been associated with amebæ, there has been some criticism of the designation because the writer did not adduce any proof of ameboid motion. It is scarcely to be expected that ameboid activities could or would come into play when the parasites occupy tissue spaces or the interior of host cells and are multiplying very rapidly. Such stages

have been studied in the warm chamber from time to time, but no mobility observed excepting in 1910, when very slight changes of form were observed in the freed parasites from the liver. At that time some of the freed parasites, shown in Plate XV., Fig. 7, both at room temperature (about 85° F.) and in the warm chamber pushed out small, finger-like pseudopodia, usually one at a time. Some of the spherical, hyaline forms became somewhat angular in outline. Unfortunately, the conditions for such examination have not been favorable, owing to scarcity of living material and the separation of laboratory from the experimental enclosures. The generic name "Ameba" was given because the parasite differed from amebæ less than from any other protozoa familiar to the writer. The literature of protozoology is sufficient evidence of the frequency and ease with which generic names are changed when new interpretations arise.

The facts recorded in this early report were gathered during one summer's work in Rhode Island in 1894. The work had to be given up and, beyond the examination of animals received at the laboratory from time to time, no further work was undertaken until 1910. The outcome of these earlier investigations was the definite proof of the existence of an infectious disease attacking ceca and liver and characterized by the presence of protozoan parasites in the affected tissues which, on account of their immense numbers and their relation to tissue elements, must be regarded as the immediate cause of the lesions. Nothing concerning the mode of multiplication of the parasite in the tissues or of any details of its life cycle was determined.

The introduction of better methods of staining since the early work led the writer since 1910 to apply the Giemsa methods to sections as well as smears prepared according to the wet method. Although such procedures were in many respects more satisfactory, they did not reveal any details which could not be made out in sections of the same material stained with Delafield's hematoxylin and eosin, eosin and methylene blue, and iron hematoxylin. The results were on the whole disappointingly small, probably because material

in precisely the right stage was not obtained. These later studies have, however, brought out the early results in much better relief and added some new data which must be supplemented with careful experiments before anything definite can be formulated concerning the life cycle of this parasite.

In the report of L. J. Cole and P. B. Hadley,⁴ the authors assimilate the parasite described by the writer with *Coccidium tenellum*, a well-known parasite of birds. That there is no ground for such a position the writer has already stated in a brief critical review of this report.⁵ Since then many cases of blackhead have been examined from different flocks with special reference to the occurrence of coccidia, but in only one flock were a few found. The reason for regarding coccidia as the cause of blackhead was probably due to the thorough infection of the Rhode Island Experiment Station with coccidia. These I encountered there in a few cases in 1894 and warned against looking upon them as producing blackhead.¹ The temptation to range two protozoan parasites occurring frequently in the same host as parts of the life cycle of the same parasite is very great. It occurred in case of the mixed infection of piroplasma and sarcosporidia in cattle, although the writer had also cautioned against this error. It is not improbable that in his studies on *Hemoproteus noctuæ* and *Leucocytozoon ziemanni*, Schaudinn brought together into the life cycle of one species, stages from several different parasites as pointed out by Novy. Cole and Hadley got some encouragement from the fact that several European text-books referred to *A. meleagridis* as a coccidium. Since none of the authors of these books had ever seen the disease or tissues containing the parasite their inference cannot be regarded as of any value.

The gross appearance of the lesions of this disease as well as the rapidity with which they develop would lead one at first sight to regard it as a disease due to bacteria. The undue infiltration and thickening of the walls of the cecum and mesentery coupled with the extensive fibrinous exudation and the necrotic liver foci all suggest some very active septic process. However, a section through any of the

involved tissues with its immense numbers of parasites shows that even protozoa may prove rapidly destructive. In order to see most clearly the parasites in their relation to the tissues, material from young turkeys should be chosen. The older and the more resistant the bird the more the reactive processes come into the foreground and the more the parasites are degenerated and within host cells. The description which follows is from tissues of rapidly fatal cases of young birds, or those killed in the height of the attack.

An examination of a section through the wall of a cecum shows the parasites in the adenoid tissue between the tubules but not in the epithelium. They may occupy the entire submucosa and many have invaded the muscular coats. Here the bundles of fibers are driven apart and the newly-made spaces filled with parasites (Plate XIII.). From here they may invade the mesentery and greatly thicken and distend it (Plate XII., Fig. 2). At this stage there is no recognizable reaction on the part of the host. The parasites simply spread through the available tissue spaces, every one of which is occupied by one or many parasites crowded together.

The rapid multiplication and dissemination of the parasites causing great distension of all the layers of the cecal wall is probably responsible for the exudation into the lumen of the tube. In one case the writer saw in sections fragments of the intact epithelium lying loose as if torn away by the outpouring coagulable fluid. It would seem as if the pressure caused by the parasites within the mucosa interfered with the circulation of blood and lymph and caused the outpour. The concentric layers of fibrin indicate several distinct discharges of fluid. The exudation results in loss of epithelium which, up to this time, remains in situ even when the rest of the mucous layer beneath is densely permeated with parasites.

The parasite under conditions of rapid multiplication is about 8 to 10 μ in diameter. The cytoplasm is fairly dense, homogeneous, and only faintly stained. The nucleus, which consists of a vesicular body about 2 μ diameter, has

a distinct nuclear membrane and nucleolar body. A centriol within this has not been made out.

Under the crowded conditions usually prevailing the parasites soon undergo degenerative changes. The cytoplasm becomes vacuolated, the karyosome disappears. Later only a few filamentous remnants of the parasite occupy the rounded space in the tissue formerly filled by it. In one form of degeneration quite frequently observed, both in the walls of cecum and liver, the parasite is represented by rather long feebly tinted bands or filaments intertwining (Plate XV., Fig. 11). Though they are suggestive of reproductive forms the writer is not convinced that they are more than forms of degeneration of the parasite, for these filaments vary greatly in thickness and probably in number from ameba to ameba. There is no indication of the presence of chromatin in the filaments. In the case of amebæ resolved into such filaments, the vesicle representing the nucleus is frequently still visible.

The very rapid increase in numbers of the parasite points to some form of multiplication within the tissues themselves. Migration of individuals into the wall from the lumen might explain the presence of parasites in the mucous membrane, but it cannot account for their presence in such large numbers in the muscular layer, in the attached mesentery, and in the liver. Forms suggesting multiplication, therefore, have been looked for, but nothing definite or convincing, like the merozoites of the malarial parasite, for example, has been encountered. There are, however, forms now and then seen which probably represent such a multiple agamic division. Parasites in this stage contain small circular ring-like bodies not over $2\ \mu$ in diameter. No morphological details have been made out even with the Giemsa stain when the sections are carried through acetone to xylol and mounted in cedar oil. In several instances, aggregations of such disc-like bodies have been found also in wet smears stained according to Giemsa. The writer is strongly inclined to regard these ring-like bodies in spite of the meager

morphological details as products of multiple agamic division. The process is probably very rapid and cases should be found in which large numbers are sporulating. Unfortunately, this must occur early in the disease, and when the young bird begins to droop the invasion is past its active stage.

In several cases there were found many host cells containing spherical bodies of various sizes, chiefly between bundles of fibers of the muscular coats of the affected cecum. The host cells are probably phagocytes and the contained bodies young parasites (Plate XVI., Figs. 12 and 13). The plate shows each cell stuffed with these bodies. They were seen in sections stained in the various ways indicated above but the Giemsa preparations were most satisfactory. The dyes penetrated into these bodies with difficulty and even after twenty-four hours' staining, some remained unstained. A tentative interpretation of these appearances is as follows: The phagocytes take in one or several parasites which multiply within them and the dissemination of the progeny fills the host-cell completely. They probably assume a protective covering or capsule which interferes with the passage of the dye.

The lesions in the liver do not differ appreciably from those in the cecum. They start from emboli of parasites or, what is more likely, of phagocytes filled with young forms which, on reaching the liver from the walls of the ceca through the portal circulation, begin to multiply rapidly and move outwardly in all directions, forming the spherical foci so characteristic of this disease. What has been stated of the parasite in the walls of cecum applies here in all details. The rapid multiplication leads to interference with the circulation, coagulation necrosis results and the focus assumes a firm cheesy consistency and yellowish color. The yellow areas consist of masses showing no structural details. They are honey-combed and contain parasites or colonies in their interstices, thus maintaining the general form of the liver trabeculæ.

The location or habitat of these parasites deserves some

discussion. One fact seems to be certain. They do not invade epithelial cells, either in the mucous membrane of ceca or in the bile ducts of liver. Although the writer has examined many sections of ceca in which the epithelium was still intact, in spite of the great invasion of the subjacent tissues, no invasion of the epithelium could be detected. In but one section from a cecum of a young turkey obtained from the Rhode Island Experiment Station, through Dr. Cooper Curtice, was the double infection clearly in evidence. A small number of coccidia were found in the epithelial cells. They were, however, wholly different in character from the amebæ in the subjacent adenoid tissue.

In the early report the writer stated that the parasite lived within the spaces of the connective or adenoid tissue. Here the roundish spaces they occupy may still be visible in fixed, stained sections after the parasite has become disintegrated either normally, following schizogony, or abnormally as a result of overcrowding or other untoward conditions. The parasites frequently appear within phagocytes and large giant cells. Are they destroyed in the host cell, do they destroy the cell, or do both parasite and host cell remain alive in certain cases and the former utilize the latter for a time? These are questions which hinge upon the existence of other still unrecognized stages of the parasite.

The grouping of the parasites within the spaces, there being from one to many in a single space, the total absence of any uniformity or regularity in the relation of parasites to the invaded tissues and the prompt degeneration and disintegration of the parasites are opposed to the view that this microörganism, at least in this stage, is normally a cell parasite. The large giant cells frequently seen in advanced stages and in older birds manifesting more or less resistance are probably only incidentally concerned in taking in and destroying parasites. Their chief function seems to be to digest and remove masses of dead tissues, around the entire circumference of which they range themselves. They are well known phenomena in avian pathology, having been described many years ago.

Although the ceca are the chief seat of this disease and are evidently the region where the tissues are first invaded by way of the digestive tract, a study of the contents has thus far yielded nothing definite. The physical condition and composition of the cecal contents vary so much between health and disease and even at different stages of disease and in different places within the same cecum that extraneous accidental parasites may be stimulated to undue multiplication in one case and suppressed in another, thus opening the way for possible misinterpretations.

In liquid feces due to diarrheal conditions as well as in ceca with fibrinous exudates, at least several species of flagellates may be present. The most frequent form is trichomonas-like and was noticed and figured by the writer¹ in 1895. These protozoa may be present in enormous numbers and fill the lumina of the tubules and the spaces between the folds of the mucosa. So far as evidence goes at present, they are not the precursors or progenitors of the tissue parasites. Leptomonas-like forms were observed in one case densely covering and adherent to the free borders of epithelial cells.

In two cases (1910 and 1914) feebly and homogeneously tinted spheres were found which were enclosed in characteristic cysts. They varied in size from 11 to 16 μ in smears prepared according to the wet method. One was found as large as 26 μ . They were probably encysted amebæ. Their relation to the tissue parasites is unknown.

When the diseased mucosa is very gently scraped, the tissue parasites appear in the scrapings and are then very easily recognized as such. It is probable that a laborious examination of the feces of a large number of animals both healthy and diseased, at different times, might, by a process of exclusion, lead to some clue as to the presence of the causal organism in the lumen of the cecum, provided the parasite passes through some stage of its cycle in the cecal contents. On the whole, nothing has so far been definitely learned by a microscopic examination of healthy as well as putrid, bacteria-laden contents of the diseased ceca beyond

the fact that the protozoan fauna is both rich and varied. Here again field experiments may aid in directing attention to new clues.

Repair has been rarely observed and then only in larger, more resistant birds. The foci in the liver from being dark brownish red, or blackish or interspersed with minute yellow masses, become paler, pinkish gray. This change is due to the infiltration of small round cells or lymphocytes. The following description from the early report (1895) refers to a more advanced stage of repair: "Within the disease focus, the liver tissue recognizable as such is present only in irregular patches of variable extent. The remainder has been replaced by an actively developing connective tissue still rich in nuclei. Within these areas there is an extensive formation of bile ducts . . ." That turkeys may and do recover from this disease is known to turkey raisers who frequently make efforts to treat the sick with various remedies. The survival of the treated birds is usually attributed to the remedy. This inference may or may not be true. A distinction should be made between very young turkeys under two months of age and older birds. The disease in the young is much more virulent than in the older birds. The mucosa of one or both ceca is usually completely destroyed and the lumen blocked with a fibrinous cast which undergoes putrefaction. Even if the turkeys could relieve themselves promptly of this firm plug of coagulated exudate, the mucous membrane could scarcely become regenerated enough to make the animal profitable. The ceca evidently serve an important function in the absorption of the digested food. The impairment of this function must permanently reduce the vitality of the bird.

In the older birds the ceca usually contain discontinuous patches of necrosis and exudation, which do not offer the same difficulties. The necrotic foci in the liver undergo absorption and are gradually replaced by connective tissue as stated above. In short, we must expect to find in this as in most other infectious disease, cases varying very much in

severity. Some are probably so mild that the lesions fail to leave any traces in the recovered bird.

Occasionally a mild, unrecognized attack of blackhead may be followed by serious consequences. In 1913 a dead turkey weighing about twenty pounds was received at the laboratory. There were no fresh lesions of blackhead. The distal two-thirds of one cecum was greatly distended and filled with a consistent, gummy, fecal mass of cylindrical outline 15 centimeters long and 5 centimeters in diameter and of considerable weight. The distended portion of the cecum was completely shut off from the proximal portion. The condition probably arose from an ulcer following an attack of blackhead which, in healing, closed the lumen. The large mass of feces was the result of the gradual accumulation of secretions, desquamated cells, etc. The immediate cause of death was bacterial sepsis, for large colonies of bacilli were found both in lungs and liver.

The presence of bacteria might be expected with such extensive destruction of tissue in ceca and liver. As a matter of fact they are rather uncommon in the liver necroses of turkeys chloroformed at the height of the disease. In turkeys which die during the night, smears and cultures may show next day several varieties of bacteria. The histological study brings strong evidence that bacteria have nothing to do with the lesions, and the frequent sterility of cultures of liver foci demonstrates it.

The existence of this protozoan disease in other species of wild and domesticated birds is a matter of considerable importance. Chester and Robin⁶ describe in one hen whitish to grayish circular spots on the liver, with ceca normal. A second fowl fed with this liver died in twenty-eight days. The liver was studded with grayish spots three to fifteen millimeters in diameter, but the ceca again were normal. A third hen, received from the same source as the first, had spots on the liver and two lesions in one cecum. Feeding negative. A careful scrutiny of their microscopic studies leaves one in doubt as to whether they actually saw the blackhead parasite, for in their drawing they show only the nuclei of liver cells and not the parasites. That the blackhead organism does occasionally multiply in fowls is proved by some material received from Dr. V. A. Moore.

The parasites were clearly demonstrable in sections of liver tissue.

Cole and Hadley⁴ give a list of species examined, but it is not possible to make out clearly which were affected with coccidia and which with the blackhead parasite. They examined guinea-fowls, ducks, pheasants, quail, grouse, pigeons, and sparrows. A critical study of the text indicates probably that the lesions of blackhead were detected in guinea-fowls and quail, and that the other species were affected with coccidia only. The suspicious lesions of intestines and liver found in pigeons "not accompanied by coccidia" may have been tuberculosis, which is not uncommon in this species.

THE TRANSMISSION OF BLACKHEAD. — Whatever theory we may entertain concerning the nature of the parasite will have to be tested in the light of experimental evidence. Unfortunately, few of the actual experiments made thus far will withstand critical examination. The reason for this will be obvious as we go along.

One group of experiments concern themselves with the attempt to produce the disease by feeding affected tissues. Following the work of the writer, V. A. Moore⁷ fed healthy turkeys about five months old obtained in the District of Columbia with the excreta and organs of diseased turkeys sent from Rhode Island. Two turkeys were fed liver and ceca of three diseased turkeys November 28, 1895. One of these died in forty-four days, evidently of blackhead. The other killed two days later was unaffected. Four healthy turkeys were penned with two diseased turkeys November 28th and at the same time they were fed the excreta of the latter for two weeks or longer. Three of the fed birds became diseased and one remained well. The time elapsing between the beginning of exposure and the death of two was twenty-two and twenty-seven days respectively. The third was found diseased when killed on the forty-sixth day.

Curtice^{2,3} carried on a number of feeding tests. The material fed consisted of the organs of young turkeys or

poults affected with blackhead. Most of the birds fed remained unaffected over a period sufficiently long to develop the disease. Some died of blackhead. Curtice does not draw any definite conclusions from these tests since they were not decisive. He was working in a heavily infected region and the source of infection could not be traced. He is inclined, however, to infer that the disease was not communicated by his feedings.

Cole and Hadley⁴ reported successful infection of young turkeys by feeding them with diseased tissues from young chicks. The reverse process was also successful. Unfortunately, it is not possible from the text to determine whether coccidiosis or blackhead is meant.

The only direct feeding experiment made by the writer is the following: Two turkeys hatched in the incubator in early June, 1913, and reared on virgin soil as regards poultry and turkeys were fed, beginning September 22d, with the chopped-up, diseased ceca of two turkeys between two and three months old. The material was offensive and, to conceal it, a special sterilized food of grain, eggs, and milk was mixed with it. The turkeys were fed on the evening of September 22d, the morning and evening of September 23d, and the morning of September 24th, each time with a mass about the size of a hazelnut. The material was kept in the refrigerator. Both remained well up to December 24th, when the enclosure was broken into and one stolen. The other, chloroformed December 27th, was normal.

These meager experiments are not at all conclusive and will have to be repeated. They involve also the question of the infection of poultry with this parasite. Curtice had previously concluded that common fowls are hosts of the blackhead parasite, although his experiments conducted under uncertain circumstances and in suspicious surroundings do not warrant definite conclusions.

The many unknown interfering possibilities that may be brought forward against any experiment in which healthy animals are exposed to sick animals, or are fed with products of disease make it imperative to remove such possibilities at

the outset. The first prerequisite is a supply of healthy animals for the experiment. In the investigation before us such animals must be obtained by artificial incubation and brooding and rearing on relatively virgin soil as regards poultry of all kinds. Curtice reared a number of poults by placing the eggs which had been incubated under hens in an incubator the three or four final days of incubation, after wiping them with a cloth moistened with ninety per cent alcohol. By these means he was able to reduce losses from blackhead very materially but not to eliminate the disease. A careful search of Curtice's bulletins does not reveal any statement that the artificial incubation and rearing of turkeys had been carried out from the start. Evidently the artificial procedures began as stated above with the last three or four days before the end of incubation by turkey, hen, or common fowl.

Inasmuch as the mere presence of adult turkeys may be a menace to the young, the writer planned to rear turkeys artificially. In the absence of proper facilities, a preliminary experiment of this kind was made in 1910 for the writer by Dr. Austin Peters, who was kind enough to hatch and rear a small flock on his own premises.

Before placing the eggs in a carefully disinfected incubator, they were thoroughly scrubbed with a brush and then placed in .5 per cent warm mercuric chloride for about thirty seconds, washed in warm sterile water and dried with sterile towels. This procedure was supposed to destroy any hypothetical parasites dried on the shells. The eggs were obtained from three different sources. Fifty-four eggs were incubated and about a dozen turkeys hatched out late in May, of which three died very soon, leaving nine. These were kept in a yard confined under a movable enclosure. Near by was a hen yard, but the hens and turkeys did not mingle. The proximity between them was, however, such that the opportunity for the transfer of infection was given. The subsequent fate of the nine turkeys is given in the following table:

TABLE I.

Blackhead among artificially incubated and reared turkeys, 1910.

Number of Turkey.	Dies.	Is killed.	Weight, etc.	Color.	Condition.	Remarks.
50.	July 5.	Bronze.	Ceca and liver affected.	
51.	" 7.	"	Ceca and liver affected.	
52.	July 9.	"	Normal.	Feet deformed.
53.	July 9.	170 grams.	White.	Ceca and liver affected.	
54.	" 10.	225 "	Bronze.	Normal.	Feet deformed.
55.	" 26.	White.	Ceca and liver affected.	
58.	July 26.	Bronze.	Normal.	Feet deformed.
56.	Aug. 26.	Female.	White.	Ceca and liver affected.	
57.	" 30.	Male, weight 2,148 grams.	Bronze.	Ceca and liver affected.	

It will be seen that of nine turkeys only three were normal when chloroformed. Two of these were killed rather early because of deformed feet. It cannot, therefore, be said definitely what would have happened if they had lived three or four weeks longer. The third lived about seven weeks. During this time four died of blackhead. This normal bird had had, therefore, ample opportunity to become infected.

The unexpected appearance of blackhead among these turkeys might be accounted for in one of several ways. The protozoa might have been transmitted in the egg. They might have been harbored by hens and conveyed in some unknown way or finally they might have been introduced by other birds accidentally alighting in the yard.

This outbreak furnished the writer the first convenient opportunity for examining the coccidia theory of Cole and Hadley. Both diseased and normal birds were carefully examined and the contents of duodenum, cloaca, and ceca searched for coccidia but not a single individual found. The great prevalence of coccidia among birds as shown by the work of Cole and Hadley renders the entire freedom of birds from this plague rather unusual. If the parasite of blackhead were only a stage in the life cycle of a coccidium, it is certainly strange that the disease can run its course in a small flock over a period of two months without revealing a single stage recognizable as belonging to a coccidium. On the other

hand, the writer has seen young turkeys four weeks old from the Rhode Island Station, in which mature coccidia cysts were easily detected in the contents of the ceca, but no signs of blackhead lesions were present.

In 1913 the attempt to raise turkeys in incubator, brooder and enclosure on a small piece of land on which no domesticated birds of any kind had been kept for eighteen years or longer was made by the writer himself. Owing to certain difficulties not at first anticipated the experiment yielded but two turkeys.

On May 13th thirty-six eggs (white Holland) were disinfected as described above and placed in a new chicken incubator heated with gas. Only five hatched out June 10th and 11th. Of the remaining eggs, six contained fully developed embryos, one a partly developed embryo, and twenty-five were sterile. Of the five poults, three died soon after. Two were successfully brooded and kept confined during the summer in an enclosure covered with chicken wire, admitting neither birds, nor small mammals. They were, however, allowed to leave the enclosure daily and roam a short distance under observation. During these short trips they obtained many insects and fed over territory (grass) visited by a variety of birds. Their food consisted of a mixture of grain, eggs, and milk thoroughly mixed together and heated in an autoclave under pressure in glass preserve jars. Later in the summer uncooked grain was added to this until over half consisted of raw, mixed grain. There were no disturbances of health noticed. The droppings of the male were at times soft, tarry, and blackish. But the general condition of the bird was normal. When the two turkeys were three and one-third months old they were fed diseased tissue of turkeys as described above with negative result.

In 1914 a second attempt was made to raise turkeys in incubator and brooder on the grounds used in 1913, this time more successfully since many of the difficulties experienced in 1913 were met at the outset. Here, again, two main objects were kept in view. First, to determine whether healthy turkeys can be reared from eggs from infected flocks; second, to expose turkeys raised in this way, if healthy, to diseased birds to learn the way in which infection takes place.

Eggs were obtained from two New England flocks. In one, the presence of blackhead was positively determined in a case sent to the laboratory; in the other, blackhead prevailed according to the owner's own testimony.

The following table gives very briefly the results of rearing and of subsequent exposure to diseased turkeys.

TABLE II.

Artificial incubation, rearing, and exposure of young turkeys to "Blackhead" in 1914.

Turkey No.	Variety.	When Hatched.	Date of Exposure.	In Infected Pen.	Final Disposition.	Result of Exposure.	Remarks.
86 .	White.	May 15.	—	—	—	—	Killed by rat June 28. No blackhead.
87 .	"	" "	—	—	—	—	Killed by rat June 28. No blackhead.
91 .	"	" "	July 8.	C	Nearly dead July 20.	Blackhead.	Chloroformed.
92 .	"	" "	" "	C	Chloroformed July 29.	Normal.	—
93 .	"	" "	" "	C	Well Oct. 24.	"	Sent to infected flock Oct. 24. Killed by accident. Viscera normal.
94 .	"	" "	{ " 9. " 27.	D } C }	" " "	"	Sent to infected flock Oct. 24.
95 .	"	" "	" 9.	C	Killed Oct. 24.	"	
96 .	"	" "	Aug. 1.	C	Well " "	"	Sent to infected flock Oct. 24.
97 .	"	" "	" "	C	Killed Oct. 3.	"	
88 .	Bronze.	June 5.	—	—	—	—	Killed by rat June 28. No blackhead.
89 .	"	" "	—	—	—	—	Killed by rat June 28. No blackhead.
90 .	"	" "	—	—	—	—	Killed by rat June 28. No blackhead.
98 .	"	" "	—	—	Chloroformed July 22.	—	Diarrhea; no blackhead.
99 .	"	" "	—	—	Chloroformed July 28.	—	Feeble, diarrhea.
100 .	"	" "	—	—	Dies July 29.	—	Diarrhea.
101 .	"	" "	Aug. 1.	C	Well Oct. 24.	Normal.	Sent to infected flock.
102 .	"	" "	" "	C	Killed Oct. 3.	"	—
103 .	"	" "	" "	C	" " 24.	"	—
104 .	"	" "	" "	C	Well " 24.	"	Sent to infected flock. Killed by accident. Viscera normal.

TABLE III.

Turkeys from an infected flock used to infect Pens C and D.

Turkey No.	Variety.	Source.	Placed with Healthy Birds.	Pen.	Killed or Died.	Remarks.
105	Bronze.	From infected flock.	July 7.	C	Dies July 13.	Blackhead.
106	"	" " "	" "	C	" " 19.	"
107	"	" " "	" "	C	Chloroformed July 27.	"
108	"	" " "	" "	C	Chloroformed July 28.	"
109	"	" " "	" "	C	Chloroformed July 28.	Normal.
110	"	" " "	" 9.	D	Dies July 16.	Blackhead.
111	"	" " "	" "	D	" " 24.	"
112	"	" " "	" "	D	Chloroformed July 27.	"

Nineteen turkeys were raised. Of these five were killed by a rat which entered the enclosure through an open door when three were twenty-three days old and two forty-four days old. In none of these were traces of disease discovered. Since a large percentage of young turkeys die within three to four weeks, the evidence is pretty strong that the parasite was not in these five. Two were chloroformed when forty-seven and fifty-three days old respectively, because of diarrhea, possibly due to lead and arsenic spray. In neither were traces of blackhead found. The eighth died when fifty-four days old, probably of the same cause affecting the preceding. No disease of liver or ceca was found. The remaining, eleven in all, were exposed to diseased turkeys or an infected yard when between thirty-four and fifty-five days old.

The result of this exposure as given below further shows that blackhead was not transmitted in these eggs. These figures are sufficient to warrant the inference that turkeys raised in incubators and on soil not contaminated by poultry of any kind are safe for experiments with blackhead virus. Confirmatory experiments are, however, needed to cover a variety of conditions. It should be emphasized that failure to

raise turkeys free from blackhead with incubator and brooder does not necessarily imply that the infection was in the egg. Until we know more about the existence of the disease in poultry and in miscellaneous species of birds which visit the grounds occupied by turkeys, such experiments should be conducted after the poults have left the brooder in enclosures to which sparrows and other birds have no access and preferably at a distance from ordinary hen yards, and, of course, from turkey grounds as well. Unless these precautions are observed, the rearing of infected turkeys artificially can have no weight at present in deciding upon the presence or absence of the parasite in the egg. It should also be stated here that Curtice had reached the conclusion that the egg does not transmit infection, although his experiments, owing to interfering factors, cannot be regarded as conclusive.

The eleven remaining artificially hatched and reared animals were penned with nine young bronze turkeys received from an infected flock. The latter weighed at time of death or when chloroformed between three hundred and fifty and six hundred grams. All but one were severely diseased. Two pens were used to prevent overcrowding. They were arranged as follows: A shelter was provided against predatory animals, birds, etc., where they were kept at night. During much of the day they were allowed to pass from this shelter into a small enclosure made by staking out chicken wire three feet high so as to shut in about two thousand square feet. This fence was shifted occasionally to provide fresh grass. In one (C), three healthy and five diseased, in another (D), two healthy and three diseased were placed. The healthy were all whites. Later as the diseased began to die, the two lots were brought together in C. Immediately after all diseased were dead or had been chloroformed and removed from the pen, the remaining six healthy turkeys (four bronze and two white) were put into pen C. The pen was purposely not cleansed. In this pen they remained from August 1st until October, when the experiment was closed. Among the eleven thus exposed, only one in pen C became diseased. This white Holland turkey was exposed July 8th and was nearly dead July 20th when

it was killed. There was found uniform thickening of the wall of one cecum from the tip to within one-third of the proximal end. No exudation. Gentle scraping of the mucosa brought out many parasites singly, grouped, and within phagocytes. The liver lesions were in an early stage; they were but faintly outlined and without yellowish necroses. Subsequently, sections confirmed the diagnosis and demonstrated the presence of an enormous number of parasites in the affected organs. One other of the exposed, appearing somewhat droopy, was chloroformed after twenty-one days of exposure, but no lesions found. Of the remaining nine, five were killed in October and found normal, and four transferred to an infected flock. Of these four, two were killed accidentally soon after. The viscera returned to the laboratory were free from disease. The remaining two have not been heard from.

This exposure experiment was remarkably unproductive of disease and does not give us any direct clues. That these birds could have been immune seems far-fetched, when we consider that one of them succumbed early to a severe attack. Opportunity for the transmission of infection to the first lot of five was very good. The turkeys roosted on a box placed six feet above the ground, where the sick and well were huddled together all night. The box was covered with a thin layer of dried alfalfa and the discharges remained in this layer three or four days before removal.

The extent of the exposure of this first lot is indicated in the following synopsis:

- No. 91. Exposed July 8th. Dies July 20th. In contact with four diseased.
- No. 92. Exposed with and to 91. Chloroformed July 29th. Normal.
- No. 93. Exposed with Nos. 91 and 92. In contact with five diseased.
Sent away healthy October 24th.
- No. 94. Exposed July 9th to three diseased, of which one dies July 16th, one July 24th. Transferred July 27th to pen C and kept there.
- No. 95. Treated like No. 94.

When the remaining six were exposed in pen C, five diseased turkeys had occupied this pen at night and fed

over the small adjoining enclosure by day. Yet no disease developed.

It is obvious that while this experiment points out the laborious way which must be followed, it does not permit us to draw any conclusions. These hinge entirely on the interpretation to be placed upon the single positive case of blackhead developed among the eleven exposed. In this one case, the infecting agent may have been brought with the young turkeys from original sources, while the virus multiplying within them may have been in a stage incapable of transmitting infection.

GENERAL SUMMARY. — The rapid course and high mortality of blackhead is not paralleled by any other protozoan disease. The lesions are apparently due as much to the mechanical disturbances accompanying the enormous invasion and the consequent distension of tissues as to any toxic action of the parasites.

The nature of the parasite remains in doubt when we attempt to range it beside known forms. There are indications that the parasites are only in small part invaders from the cecum. The larger number are probably the result of agamic or schizogonic multiplication within the tissues. Just how this multiplication takes place is unsettled. Most probably the disc-like bodies which have been seen under a variety of conditions within the amebæ represent the agamic progeny. These have been counted up to sixteen in a single individual.

This organism is not a cell parasite in the usual acceptance of the term, at least not in the stage of active multiplication and dissemination through the invaded tissues.

The invasion of the liver is through the blood. The parasites are transported either free or in phagocytes from the affected ceca and, lodging in the sinusoids, multiply as in the walls of the cecum without invading the liver cells themselves. Here also the rapid increase in numbers causes disturbances which lead to necrosis of liver cells in characteristic foci. Thus far, the organism has not been detected in

tissues other than those mentioned. The evidence is pretty conclusive that the microörganism is not a secondary invader but a primary sufficient agent in starting and maintaining the disease.

Putting together the data at hand, certain facts seem to stand out quite clearly. The parasite, from the fact of its destructive effect on the young bird's life, is poorly adapted to its young host. The process of invasion into the walls of ceca and liver is not adjusted to the discharge of parasites for passage to another host. The parasites are buried within the host lesions. Again the cycle as observed is obviously incomplete. There is all told a remarkable want of adaptation of means to ends such as we find so fully developed in the coccidia and protozoan blood parasites, for instance. The evidence points to several possible theories rather widely divergent. The disease may represent a kind of aberrant parasitism, the true host being some other species. Or the parasite may undergo its normal development in the contents of the ceca and the invasion of the tissues may be abnormal. Or there may be still other stages and an intermediate host. These views can only be definitely proved or disproved with the aid of the experimental method. The writer does not feel committed to any one of these hypotheses. The results obtained on feeding in 1913 and on exposing young artificially reared turkeys to young diseased turkeys in 1914, were not definite enough to prove that infection is direct from diseased bird to healthy, and they will require repetition and amplification.

In casting about for a satisfactory method for raising trustworthy birds for experimental work the writer found that healthy turkeys could be reared from infected flocks by using the incubator and brooder. This procedure has made it fairly evident that blackhead is not transmitted in the egg, although more trials are needed before we can be certain of this. Pending the definite disclosure of the life cycle of this parasite, the suggestions which can be given to those who are raising turkeys must necessarily be tentative and perhaps more comprehensive than may eventually prove necessary.

The one way which seems to cover all possible life cycles of the parasite is the one tried by the writer. Turkeys should be raised artificially in incubator and brooder and those that survive should be used for breeding stock. The land should be free from older turkeys and fowls, at any rate since the preceding fall. If a flock raised in this way should contract blackhead, attention must be paid to the commoner visiting birds. A knowledge of the existence of blackhead parasites in other species of domesticated and wild birds is, therefore, of great practical importance. If the infection can be successfully eradicated from turkey flocks, but continually reintroduced from other species, the labor of purifying turkeys alone is in vain. Our information on this phase of the subject is as yet exceedingly meager and unsatisfactory.

Those who would wish to incubate the eggs under hens up to the three or four last days, and then transfer them to an incubator, should be warned to carefully disinfect the shell with warm solutions as recommended above before placing them in the incubator. Such incubation should be done on an adjoining farm and the young transported to land free from turkeys or poultry. If the incubation is to be carried out in the incubator, the eggs should be placed in it as soon as possible after they are laid to prevent drying out, and the incubator should be kept thoroughly humid, otherwise the young will have deformed feet. One drawback noticed by the writer during his limited experience is the tameness of the birds raised artificially. It is probable, however, that the offspring of these birds when reared in the natural way will possess the usual shyness and alertness needed to protect them from injury.

In conclusion, it should be stated that the artificial incubation and rearing of turkeys for breeding stock eliminates not only the blackhead parasite but also a variety of other parasites both internal and external. The young turkey starts life without any parasites. To what extent its body may be restocked in the long run from other species of birds remains to be determined.

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DESCRIPTION OF PLATES XII.-XVI.

(Owing to unavoidable obstacles, the writer has been unable to have drawings of the parasite prepared. These will be furnished in a later paper.)

PLATE XII., FIG. 1.—Lymphoid nodule in wall of cecum of healthy turkey seven months old. $\times 15$. Note the ramifications of the epithelium within two (probably one) cavities in the nodule. These communicate with the lumen of the cecum through a narrow neck as shown in Plate XIII. This section serves to a certain degree as guide in estimating the amount of thickening of the wall of cecum due to the invasion of parasites as shown in Plate XIII.

FIG. 2.—Cross-section of both ceca and the small intestine to which they are attached by mesenteries. $\times 5\frac{1}{2}$. Note the very great thickening of the wall of the cecum on the right and the lesser thickening of the cecum on the left. The mesentery attaching the tube on the right to the small intestine below and between them is enormously swollen by the invasion and multiplication of parasites. The wall of the small intestine is, however, not invaded. The normal mesentery attaching the cecum on the left is shown by a few shreds of tissue in cross-section.

PLATE XIII., FIG. 3.—Transverse section through the wall of one cecum of a diseased turkey about four weeks old. $\times 25$. Note the great thickening of the wall due to the invasion of the submucous and muscular layers with parasites. The whitish areas in the mucosa on the left extending almost to the free border are masses of parasites. The mass above in the lumen represents exudates mixed with fecal contents. On the right is a mucous crypt with its opening to the surface, the whole embedded in lymphoid tissue.

FIG. 4. — Transverse section of one cecum of a diseased turkey about eight weeks old. $\times 45$. The section has been cut through a mucous crypt and its opening. The entire tissue is infiltrated with parasites, some of which can be outlined as minute roundish bodies.

PLATE XIV., FIG. 5. — Section of liver of turkey about six to eight weeks old. Eosin and methylene blue. $\times 1000$. The center of the figure is occupied by a group of parasites evidently within some phagocytic host cell. Two of the bodies show the nuclear membrane and a large nucleolar body or karyosome.

FIG. 6. — Section of liver of another turkey (same as one from which Fig. 4 obtained). Eosin methylene blue. $\times 1000$. A large group of parasites, some showing the nucleus distinctly, some degenerating.

PLATE XV., FIG. 7. — A bit of diseased liver of turkey No. 57 (Table I.) teased out, fresh, immediately after the animal had been chloroformed, and photographed. $\times 500$. The dark oval bodies seen chiefly on edge are red corpuscles. The finely granular material is from disintegrated liver cells. The parasites appear among the tissue cells free. They are perfectly round, pale. The specks and granules on them are chiefly from the crushed tissue cells. They vary somewhat in size. No morphological details to be made out. Near the lower left hand corner is a group of three hardly to be distinguished as more than one large body and probably within a phagocyte.

FIG. 8. — A group of parasites near areas of necrosis. $\times 1000$.

FIG. 9. — Parasite containing disc-like bodies. $\times 1000$. Not well brought out.

FIG. 10. — Another parasite containing disc-like bodies. Iron hematoxylin. $\times 1500$.

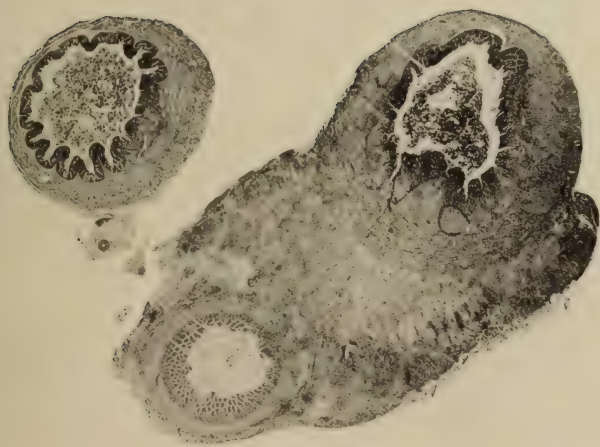
FIG. 11. — Space occupied by parasite containing a number of band-like, interlacing, filamentous bodies. Giemsa stain. $\times 1500$. Probably a degenerative stage.

PLATE XVI., FIG. 12. — Transverse section of wall of cecum of a diseased young turkey. $\times 1000$. Giemsa stain. In the upper segment are muscular fibers. Below these a number of cells are seen containing spherical bodies. The former are tentatively interpreted as phagocytes containing broods of young amebæ, the result of multiple agamic division of parasites in the tissue spaces or in the phagocytes themselves.

FIG. 13. — Another field of the same section. $\times 1500$. Giemsa stain. In the central part of the photograph, the young parasites within cells are stained; in the lower parts, the cells are filled with vacuole-like bodies. These are young unstained parasites. The difficulty with which they are stained suggests some impermeable capsule developed within the phagocyte.

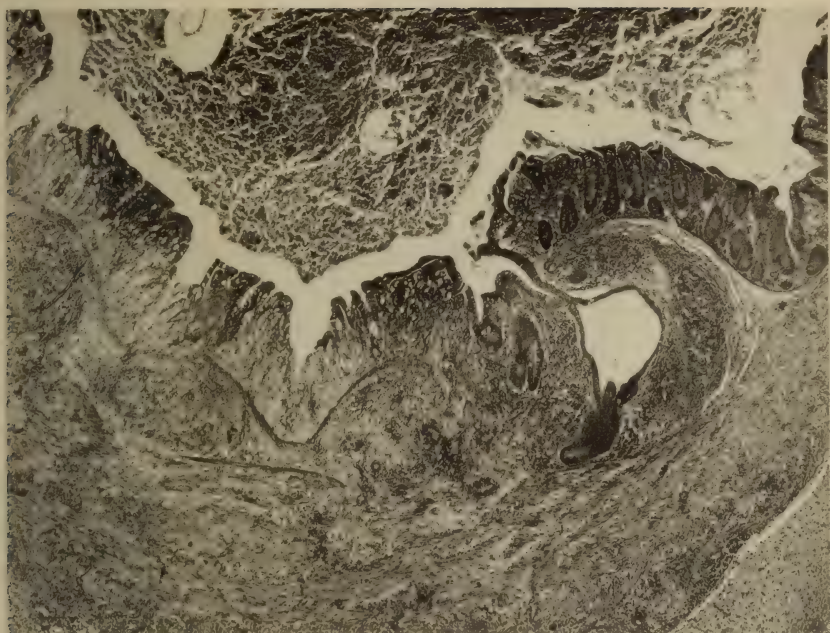


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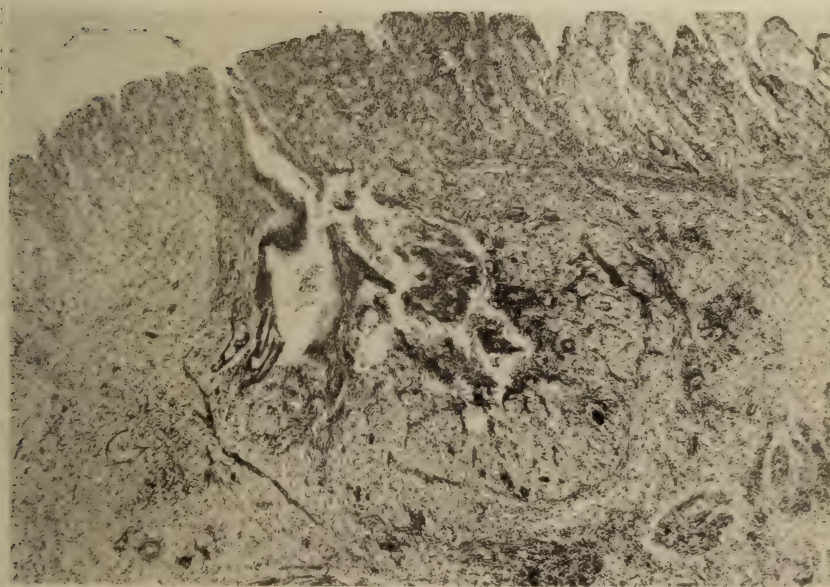


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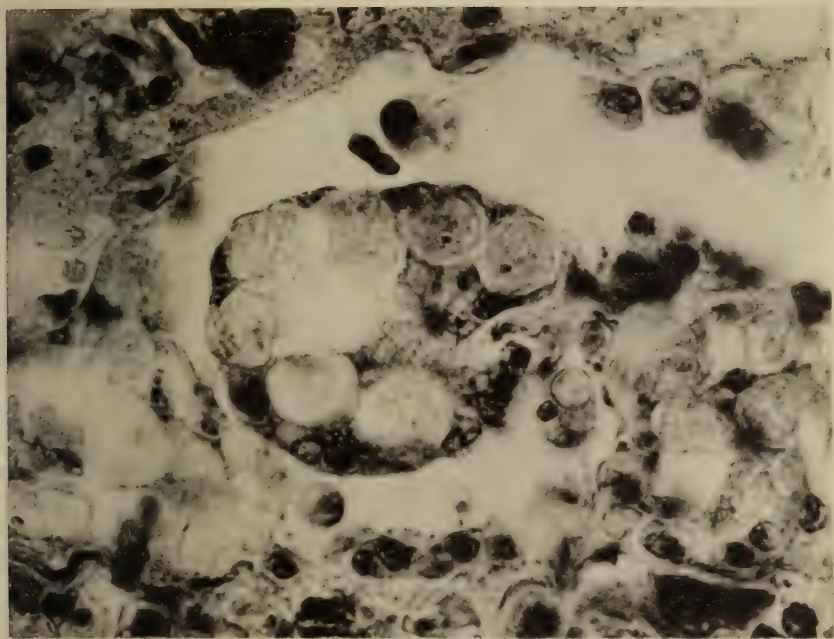


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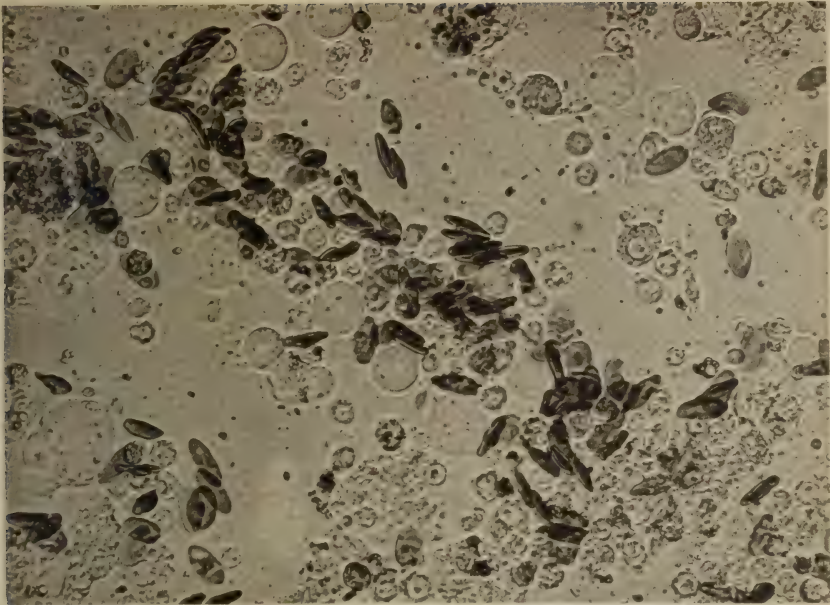


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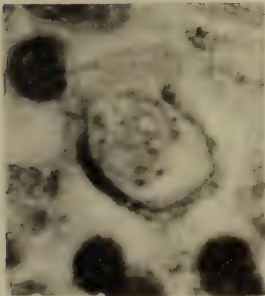


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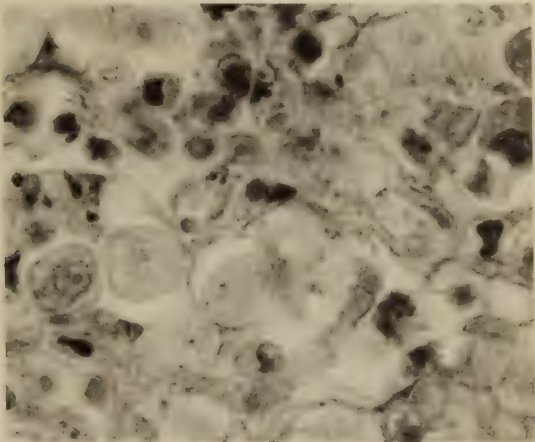
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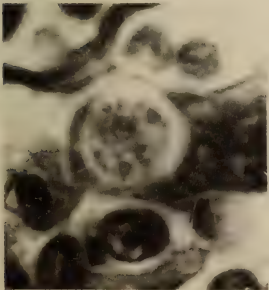
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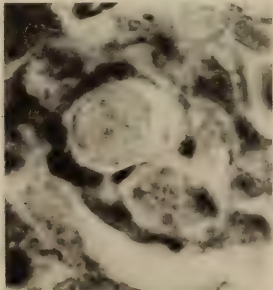
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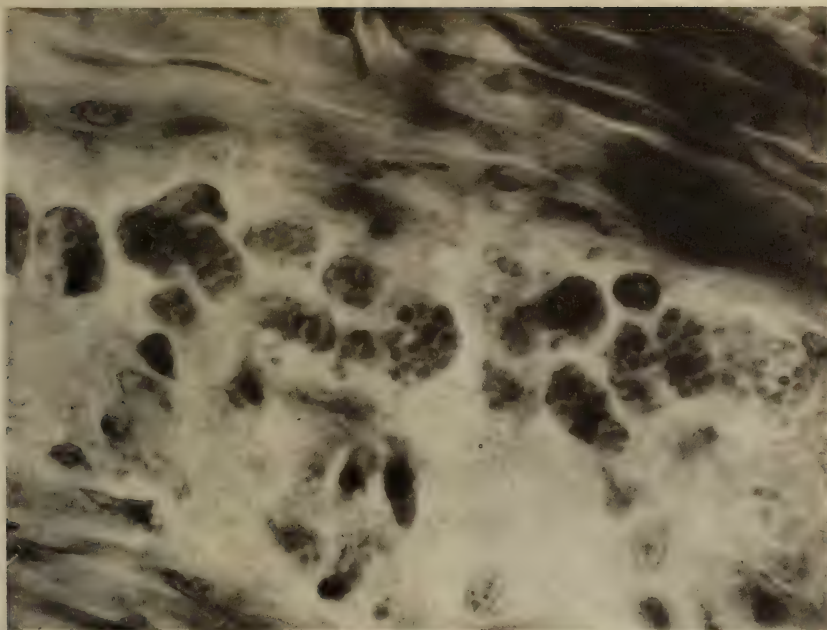


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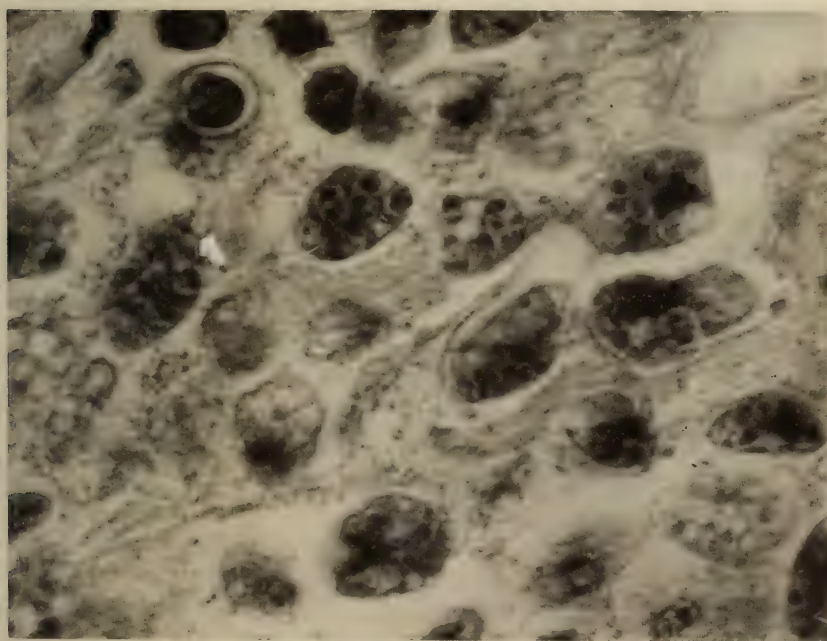


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THE BLASTOPHTHORIC EFFECT OF CHRONIC LEAD POISONING.*

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1. Introduction. Clinical and Experimental Observations from the Literature.

It is now well known that certain disease conditions deleteriously affect the germ plasm of both the male and the female of the human species. This blastophthoric effect, even though it may occur in some infectious diseases such as typhoid fever, is quite apart from the transference of the infecting agent to the germ cells. In fact, it has been convincingly demonstrated that toxic substances of non-infectious origin, notably alcohol, are equally capable of producing a blastophthoria of a marked degree.

Since this type of injury implies the possibility that purely external and extrinsic etiological factors may so work upon the germ plasm that the resulting changes are manifest as intrinsic disease, the recognition of the agents producing such changes becomes of the greatest importance from the standpoint of practical eugenics. Unless the injury to the germ plasm is of such a character that the perpetuation of the line is prevented or limited through its own feebleness, it is reasonable to expect that the intrinsic change wrought in the germ plasm may be shown by a departure from the normal through many generations. Such a blastophthoric effect has been attributed to chronic lead poisoning. In this case the problem assumes additional interest since it deals with one of the most common forms of occupational disease. If it can be shown that the clinical observations of lead blastophthoria are supported by experimental evidence an additional argument of unusual weight will be added to the

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many excellent reasons already existing for considering this one of the most serious of the occupational diseases. The injurious effect of plumbism will then be measured as much by the deterioration of the family line as by the impairment of the health of the individual.

In 1860 Constantin Paul¹ stated that lead poisoning of the mother, or even of the father, often results in the stillbirth of the fetus; and that the children of such parents, when born alive, usually die in the first three years of life and have but feeble resistance to disease. His attention was called to this disastrous effect upon the progeny of those suffering from chronic lead poisoning by the case of a woman who had been delivered of 3 living and healthy children previous to exposure to lead. Subsequently, she became pregnant 10 times and these 10 pregnancies were terminated by 8 abortions, 1 stillbirth, and 1 child which was born alive at term but died at the age of 5 months.

From this and many similar cases Paul accumulated extensive data in regard to the effect upon the offspring of lead intoxication of the mother. These figures have been frequently quoted in works upon Occupational Diseases, but are well worth repeating here. Of 141 pregnant women, working in lead, 82 aborted, 4 had premature births, 5 had stillborn infants and 50 brought living children to term. Of these 50, however, 35 died in the first two years of life, so that from 141 pregnancies but 15 children survived to the third year.

More important still as indicating a true lead blastophthoria are Paul's observations upon the progeny of fathers suffering from lead poisoning. He found that 32 pregnancies in 7 women whose husbands were lead workers and had shown signs of lead poisoning gave 11 abortions, 1 stillbirth, and 20 living children. Of the 20 living children, however, 8 died in the first year, 4 in the second year, 5 in the third year, and 1 after the third year. Thus but two individuals remained alive as the product of 32 pregnancies. As a result of these observations Paul concluded that the influence of lead as transmitted to the offspring from the father is just as real as when derived from the mother, but is perhaps a little less deleterious, the reason for this being that in the mother the intoxication produces its effect upon the organism not alone at the moment of conception, but also throughout the duration of pregnancy.

Sir Thomas Oliver² reports an epidemic of stillbirths in a Yorkshire town which called for an unofficial inquiry. The cause was found to be the contamination by lead of the drinking water supplied the town. With the removal of the cause, the effect ceased.

He also quotes Dr. George Reid's³ statistics upon the relationship between lead poisoning and miscarriages, stillbirths, and early death of infants. These figures are as follows:

For 100 mothers, engaged in housework,	43.2	miscarriages and stillbirths.		
For 100 mothers, engaged in millwork, not lead,	47.6	"	"	"
For 100 mothers, lead workers before marriage,	86.0	"	"	"
For 100 mothers, lead workers after marriage,	133.5	"	"	"
For 100 mothers, father a lead worker,	48.0	"	"	"

In regard to early death of infants :

Deaths under 1 year per 1,000 births :

Mothers engaged in housework,	150
Mothers, mill workers, not lead,	214
Fathers alone working in lead,	189
Mothers working in lead before marriage,	157
Mothers working in lead after marriage,	271

Oliver likewise verified the observations of Chyzzar⁴ upon the conditions in the home potteries of Hungary. In some villages he found practically no children, while the inferior physical and mental development of the offspring of these pottery workers led him to conclude that infantilism, idiocy, and mental weakness were some of the effects of "inherited plumbism." Deaths from hydrocephalus, acute meningitis, and convulsions were frequently noted among these children.

Oliver found that of the children of paint grinders 40 per cent die of convulsions during the first year of life.

Thompson⁵ has collected many of the important observations in regard to lead blastophthoria, including those of Paul and Oliver. He refers to the fact that lead has been found in the placenta and in the kidneys and liver of stillborn children of mothers who were lead workers. He quotes also from the French Department of Labor Report on Industrial Poisons, made by M. Tardieu in 1905, who found that of 1,000 pregnancies in lead workers 608 terminated in abortion; and also from P. Rudeaux,⁶ who states that out of 442 pregnancies among the wives of 75 lead workers, there were 66 abortions and 241 miscarriages.

Goodby⁷ concludes that there is little evidence for supposing that a male lead worker is less likely to beget children, or that his children are more likely to be unhealthy than those of men working in any other industrial process, provided that modern methods are employed to prevent absorption. "In the absence of any precautions whatever as to daily absorption of dangerous dust, the effect on the offspring, even in the case of male lead workers, may well be evident."

The experimental evidence of a blastophthoric effect in chronic lead poisoning has not been abundant. The results obtained by Oliver² working with fowls and rabbits have been much quoted.

“If fowl's eggs be incubated after painting the shell of some of them with a strong solution of lime and the shell of others with a solution of lead nitrate, I found that, while all those that had been painted with lime came to maturity, from not one of those painted with lead nitrate did there come forth a live chick. On opening the egg, the embryo was always found to have reached a fair stage of development. . . . Similarly in the case of pregnant rabbits, to whom lead was administered in food, miscarriage took place, and in the internal organs of the fetuses lead was found on chemical examination.”

Thompson⁵ quotes the work of Charron, who investigated the possibility of chronic lead poisoning from deep chrome yellow and bright red papers found to contain lead. Pregnant guinea-pigs, confined in cages lined with these papers, aborted and the fetuses contained traces of lead in their organs.

Legge and Goadby⁷ state that all the pregnant animals to which they gave lead aborted, and that experimental results confirm the well-known abortifacient action of diachylon. They refer to similar results obtained with guinea-pigs by Glibert.⁸

As the series of breeding experiments which forms the second part of this report was being concluded the work of Cole and Bachhuber⁹ appeared, constituting the most important experimental contribution to the subject of lead blastophthoria up to that time. They administered lead acetate, mixed with sugar of milk and fed in gelatin capsules. Animals thus poisoned were bred by a system of double mating such that the offspring by means of color or other characteristics could be identified as derived from either a normal or a lead-poisoned male. Rabbits and fowls were used and in each case their results led to the conclusion that the offspring produced by males which had been poisoned by the ingestion of lead acetate into the alimentary tract were of a lower vitality and that in the case of the rabbits such offspring were distinctly smaller in average size than the normal offspring of unpoisoned males.

Much of the clinical and experimental evidence is suggestive rather than demonstrative of a lead blastophthoria. In many cases one cannot rule out the effect upon the embryo in utero of lead intoxication in the mother as an important factor apart from and in addition to the injury to the germ plasm of the parent. Likewise, acquired plumbism may explain some of the results seen in the children in the home potteries. But with due allowance for such cases the evidence seems highly in favor of a true lead blastophthoria. The observations of Paul and Rudeaux upon the offspring of lead-poisoned fathers and the experimental work in which lead was administered to the male animal are especially

valuable as offering an apparently incontrovertible line of evidence.

2. Breeding Experiments.

In an attempt to prove or disprove the occurrence of a true blastophthoria in chronic lead poisoning, a series of breeding experiments extending over a period of eighteen months was undertaken. The changes in the male germ plasm in particular were investigated as being of the greater value in this connection, since, when the injury is directed toward the male element alone, the possibility of injury to the developing embryo through pre-natal poisoning or through impaired nutrition growing out of its implantation in the body of a diseased female does not exist. Accordingly, the general method of attacking the problem was to produce a condition of chronic lead poisoning in male animals, to breed these males to normal females and to compare the results of these matings with those of control animals under similar housing and feeding conditions. In addition, female animals were also poisoned with lead and bred to normal males in order to obtain data for comparison.

Administration of lead and determination of dosage. — In order to duplicate as nearly as possible the most common method of acquiring saturnism as an occupational disease, commercial white lead was chosen as the form to be administered. This was given to guinea-pigs by mouth in weighed doses in No. 5 gelatin capsules. Very little difficulty was met with from this method of administration and it seemed quite necessary to employ it in order to have a more definite dosage than is possible when the poison is mixed with food. The capsules, moistened with water, were placed in the animals' mouths by means of a small glass tube fitted with a glass rod plunger. Usually the capsules were masticated before swallowing and only occasionally were they rejected.

In preparation for filling the capsules, a known weight of commercial white lead was brought to a paste of proper consistency by mixing it with linseed oil and corn starch. The

weight of this mixture was then obtained and the capsules were also weighed before and after being filled with it. From this data the average dose of commercial white lead was computed. For instance, in preparing a certain batch of capsules 27.25 grams of commercial white lead were mixed with enough linseed oil and starch to make a total weight of 65.45 grams. With this paste, two hundred and fifty No. 5 gelatin capsules were filled. These weighed 7.57 grams when empty and 34.50 grams when filled. The two hundred and fifty capsules therefore contained 26.93 grams of the lead paste which was $\frac{27.25}{65.45}$ commercial white lead. The average dose of commercial white lead for this batch of capsules was therefore $\frac{1}{250} \times \frac{27.25}{65.45} \times 26.93 \text{ grams} = .045 \text{ gram}$.

The variation in the body weight of the guinea-pigs was found to be the most satisfactory guide to the intensity with which the white lead was administered and the size and frequency of the dose were changed accordingly. Care was taken that there should be no marked loss of weight in the pigs under observation and any such drop in weight was taken to be the indication for reducing the size of dose, increasing the interval between doses or temporarily suspending the administration of lead. This precaution was taken to avoid the possibility that an apparent lead blastophthoria might in reality be due to the impaired nutrition and cachectic state of the pigs if the poison were pushed too severely. That this was not the case is shown by Figures 1 and 2, which give a graphic comparison of body weight and lead dosage for a period of about five months in two representative lead poisoned guinea-pigs. It will be seen that one, a young growing female, gained two hundred and fifty grams during that period and that the other, a mature male, weighed sixty grams more after five months than when the lead was first given. In some cases there were sudden reductions in weight such that no lead could be administered for several weeks. In a few others, epileptiform convulsions were induced even when there had been no loss of weight and following the convulsive attacks such pigs were so ill

LEAD FEMALE 3.

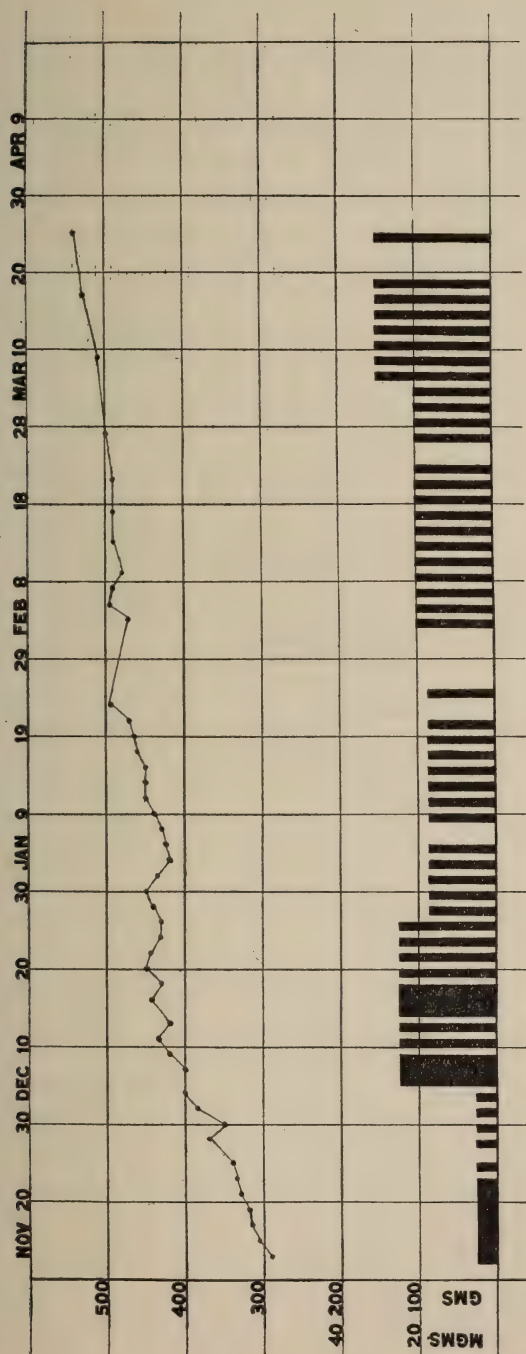


FIG. 1.

Graphic comparison between the weight curve and the dosage of white lead in a young growing female. A gain of 250 grams is shown in spite of the administration of 66 doses of white lead ranging from 5 to 30 milligrams each.

LEAD MALE 11.

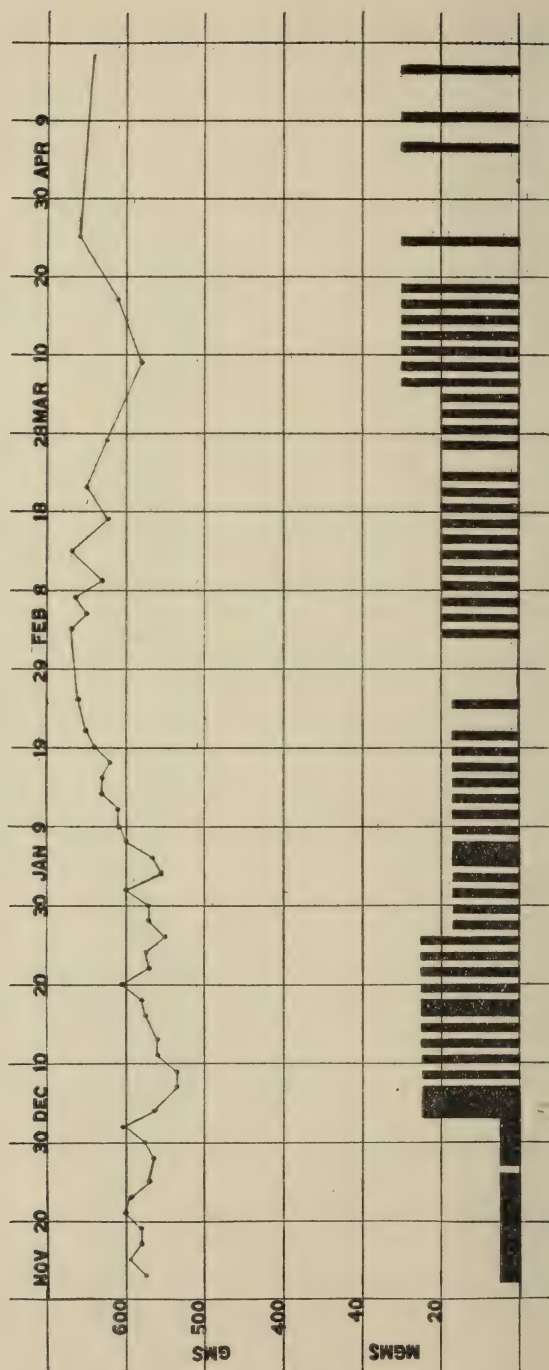


FIG. 2.

Graphic comparison between the body weight curve and lead dosage in an adult male guinea-pig. A gain of 60 grams is shown in spite of the administration of 75 doses of white lead ranging from 5 to 30 milligrams each.

that no lead was given for several days. With these exceptions lead was administered every other day throughout the greater part of the period covered by the experiment. Undoubtedly, had the lead been pushed more vigorously, the injury to the germ plasm would have been more severe and the results of breeding experiments more spectacular, but conclusions as to the blastophthoric effect of lead, *per se*, could then be drawn with much less certainty.

The amount of commercial white lead given at each administration was made to vary from six to eighty-two milligrams. The following table shows the number of doses, the period over which they were distributed and the total amount of commercial white lead received by a few pigs selected as representing the general character of this phase of the experiment.

TABLE I.

	Number of Doses Received.	Duration of Administration.	Total Amount of Commercial White Lead Received.
Female G.P. No. 3.....	95	11 months.	2.310 grams.
“ “ “ 12.....	106	11 “	2.479 “
Male “ “ 11.....	109	9½ “	2.660 “
“ “ “ 17.....	52	3½ “	1.163 “
“ “ “ 26.....	129	12 “	4.679 “
“ “ “ 44.....	91	8½ “	3.992 “
“ “ “ 50.....	95	8 “	4.016 “

Matings. — Guinea-pigs thus chronically poisoned with lead were mated, lead females with normal males and lead males with normal females. In addition, a series of matings of normal males with normal females was maintained at the same time and under the same housing and feeding conditions as for the lead-poisoned pigs. An additional check upon the results was obtained by subsequently breeding the normal females, which had given birth to offspring by lead-poisoned males, to normal males. Thus we were enabled to

rule out the possibility that an apparent blastophthoric injury to the male parent was but an expression of an intrinsic fault in the germ plasm of the supposedly normal female. Some of our most interesting results were obtained by means of this method of breeding a female alternately to a lead-poisoned and to a normal male.

Summary of results of breeding experiments. — A total of ninety-three matings yielded one hundred and seventy offspring. The distribution of these in the various groups and the average birth weight of the offspring in each group are shown by the following table :

TABLE 2.

	Matings.	Offspring.	Average Birth Weight.
Free male with free female	32	58	81.5 grams.
Lead male with free female	34	65	66.3 “
Free male with lead female	27	47	69.3 “

Thus it will be seen that the offspring of lead-poisoned fathers fell, on an average, 15.2 grams below the normal birth weight, and that the offspring of lead-poisoned mothers averaged 12.2 grams below the normal birth weight. That the difference in the latter case is not greater than 12.2 grams is doubtless due to the fact that in most cases no lead was given the female pigs during the nursing period, for we were interested in the rate of growth of the offspring and did not care to introduce the factor of a possible poisoning of the young through the milk. As a result, the administration of lead to the female pigs was necessarily somewhat irregular and some of them conceived at a time when they were comparatively free from the poison. As shown by individual cases, when lead was administered continuously to the female pigs the birth weight was reduced to even a greater degree than when the father was lead poisoned.

In addition to the average birth weight, the number of stillborn offspring and the number dying in the first week after birth were observed as additional points which might indicate a possible injury to the germ plasm. In the table which follows these data are given for the offspring in the various groups:

TABLE 3.

	Offspring.	Stillborn.	Died First Week.
Free male with free female	58	3	2
Lead male with free female.....	65	3	9
Free male with lead female.....	47	8	0

It will be noted that there were eight stillborn out of forty-seven offspring of lead females as compared with three stillborn out of fifty-eight offspring of control pigs and that nine out of sixty-five offspring of lead males died during the first week as compared with but two out of fifty-eight control pigs.

The results obtained in certain guinea-pig families by breeding the mother alternately to lead-poisoned and to normal males may also be conveniently shown in tabular form.

TABLE 4.

Offspring of Normal Female No. 36.

By lead male No. 11	{	69 grams.
		65 "
By free male No. 9	{	91 grams.
		90 "

It will be noted that the difference between the average birth weights in these two litters is 23.5 grams.

TABLE 5.
Offspring of Normal Female No. 57.

By lead male No. 50	{	54 grams.
		47 "
		40 "
By free male No. 9	{	79 grams.
		79 "

It is evident that the difference of thirty-two grams between the average birth weights in these two litters is much greater than can be accounted for by the difference in the number of offspring in the two cases. Of the three dwarfed descendants of the lead father the smallest died on the fourth day, but the other two grew to maturity in spite of the fact that they were but little more than half normal size at birth.

TABLE 6.
Offspring of Normal Female No. 42.

By lead male No. 11	{	51 grams.
		37 "
By free male No. 9	{	105 grams.
		101 "
By free male No. 9	{	75 grams.
		70 "
		62 "
		52 "

In the third litter of four pigs with a normal father, the smallest weighed more than the larger of two pigs in the litter produced by a lead-poisoned male.

TABLE 7.
Offspring of Normal Female No. 29.

By lead male No. 26	70 grams.
By lead male No. 50.....	57 grams.
	48 "
	46 "
By free male No. 10	90 grams.
	75 "
	75 "

The last two litters produced by normal female No. 29 are especially good for comparison since the number of offspring was the same in each. For the same reason Table No. 8 showing the birth weights of the offspring of Free Female Guinea-pig No. 20 is included here. From this pig there were four litters, two of two each, and two of four each. Thus differences in birth weights due to the varying numbers in successive litters could be eliminated.

TABLE 8.
Offspring of Normal Female No. 20.

By lead male No. 17. (Had received lead for but two weeks, practically a normal male)	{	65 grams.
		60 "
By lead male No. 11	{	43 grams.
		41 "
By free male No. 9	{	77 grams.
		76 "
		71 "
		66 "
By lead male No. 50	{	59 grams.
		49 "
		46 "
		38 "

It must not be supposed that all the results obtained were as uniformly consistent as in the five groups presented in Tables 4 to 8. If such had been the case the summaries presented in Tables 2 and 3 would have been even more conclusive, but they include all of the pigs born during the course of this experiment and therefore contain such irregular cases as appear in the following tables:

TABLE 9.
Offspring of Normal Female No. 15.

By normal male No. 10.	87 grams.
By lead male No. 26.	76 grams.
	70 "
By lead male No. 26.	75 grams.
	55 "
	53 "
By lead male No. 26.	112 grams.
	95 "

TABLE 10.
Offspring of Normal Female No. 19.

By a normal male	85 grams.
By lead male No. 17.	84 grams.
	80 "
By normal male No. 9.	70 grams.
	66 "
	63 "

LEAD MALE 26.

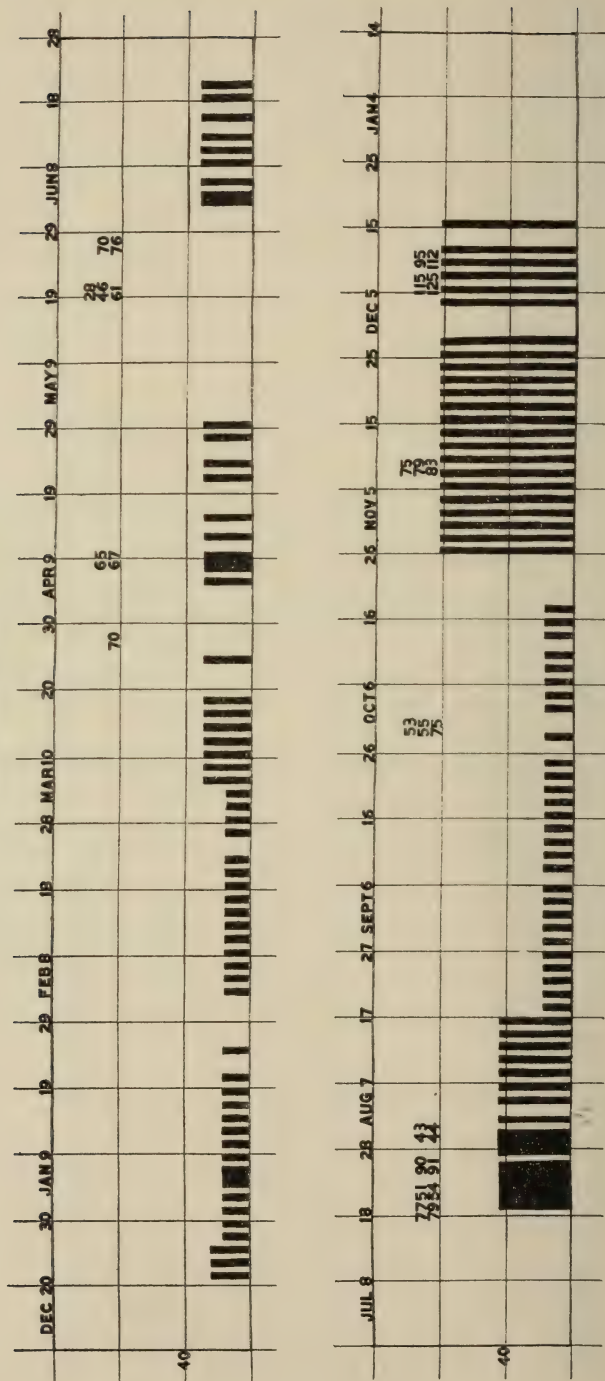


FIG. 3.

Graphic representation of the relationship between lead dosage and weight of offspring for Lead Male Guinea-pig No. 26. The dosage of lead is in milligrams and the birth weights of all offspring are indicated in grams and are set back to the probable date of conception.

LEAD MALE 11.

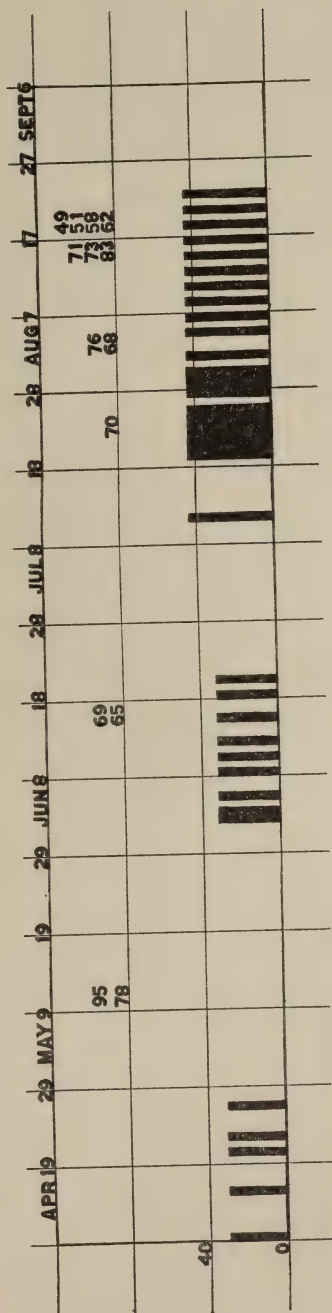
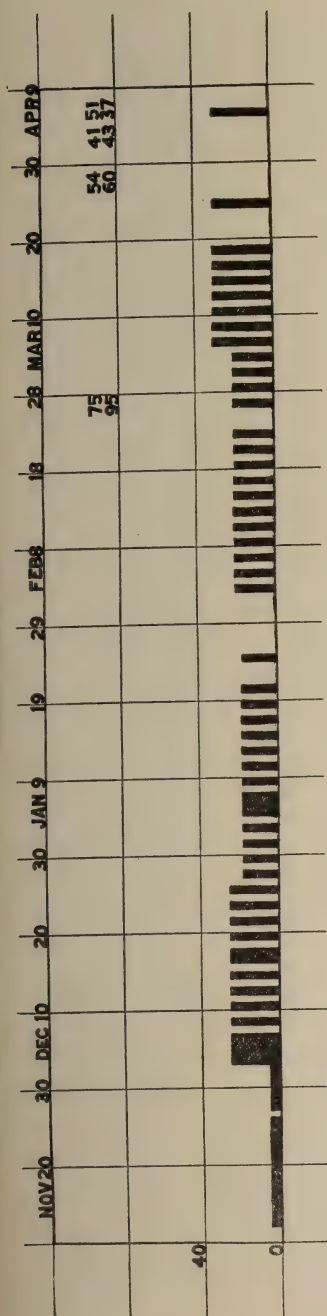


FIG. 4.

Graphic representation of the relationship between lead dosage and weight of offspring for Lead Male Guinea-pig No. 11. The dosage of lead is in milligrams and the birth weights of all offspring are indicated in grams and are set back to the probable date of conception.

The extent to which such irregular results appeared can best be shown by presenting graphically the entire history of some of the lead-poisoned males. This is done in Figures 3 and 4, in which the complete data as to lead dosage and birth weights of offspring are presented for Lead Males Number 26 and 11 respectively. The daily dosage in milligrams of commercial white lead is indicated by the solid black columns, and the birth weights of the offspring are indicated in figures, which are set back so as to appear at the probable date of conception instead of the date of birth. Recalling that the average birth weight of normal pigs in our series was 81.2 grams, it is noteworthy how many of these weights fall far below that standard, but an interesting exception is found in the last two litters produced by Lead Male No. 26, which were conceived at a time when he was receiving a relatively heavy dosage of lead.

Discussion of results of breeding experiments.—In general, no marked reduction in fertility is shown by the results of breeding lead-poisoned guinea-pigs. Notwithstanding the fact that in several instances lead-poisoned males failed for three or four months to impregnate any of the females with which they were running, the average number of offspring per mating in this group is practically the same as in the group of normal pigs. Only one of the lead-poisoned males proved absolutely sterile. Lead Male No. 44, weighing five hundred grams when lead was first administered, failed to impregnate any of the five adult females with which he was caged for eight months. During this period there was no apparent loss of sexual desire or sexual activity. All of these females proved fertile when a normal male was substituted for No. 44. Likewise, in the female there was no loss of fertility.

The birth weight of the offspring proved to be the factor which was most consistently affected by chronic lead poisoning in the parents. In normal pigs the weight of the young at birth is varied to some extent by the age of the mother, but more so by the number of young in each litter and by

the individual habit of the female, since some animals, regardless of the male parent, always produce young much below the average in weight. The effect of the first two of these factors has been overcome by the comparatively large number of observations from which our averages were obtained, while the method of breeding alternately to lead-poisoned and to normal males serves to control the intrinsic variations in the females. With these precautions, a reduction in the weight of the offspring of lead-poisoned males of 15.2 grams, equal to 18.6 per cent of the average birth weight of normal pigs, can be attributed only to the blastophthoric effect of the lead.

WEIGHT CURVES.

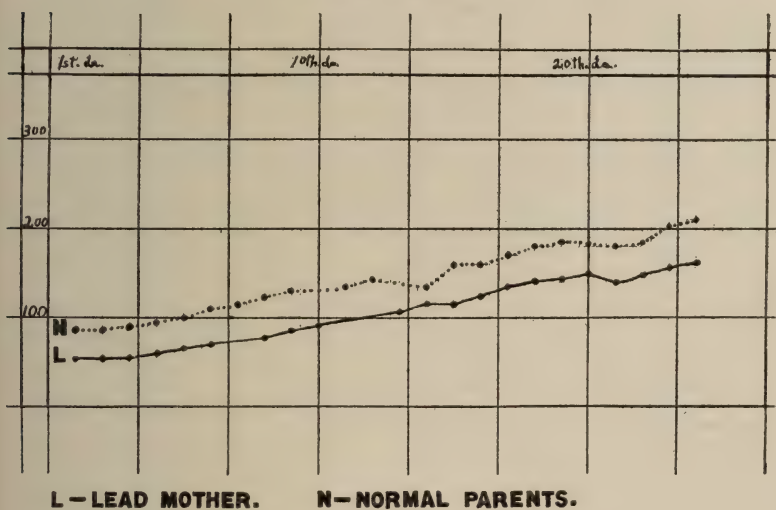


FIG. 5.

The weight curves of two newborn guinea-pigs. The one from a normal mother is indicated by a dotted line, the one from a lead-poisoned mother by a solid line. The lead offspring remains permanently underweight.

Upon the maternal side, the reduction in birth weight does not lend itself as a proof of blastophthoria. However, it is a very significant fact that the offspring of female guinea-pigs, chronically poisoned with lead in such a slight degree

that they did not themselves show any ill effects, weighed on the average 12.2 grams, equal to 14.9 per cent less at birth than did the offspring of normal pigs.

Those offspring of a lead-poisoned parent which are decidedly below the average weight at birth remain permanently underweight as compared with normal pigs of the same age. In Figure 5 are shown the weight curves of two young growing pigs, one with normal parents and one with a lead-poisoned mother. Although the latter more than doubled its birth weight in twenty-four days, at the end of that period it was fifty grams smaller than the guinea-pig born of normal parents.

Stillbirths are not common among normal guinea-pigs, nor were we able to show that lead intoxication of the male parent results in any increase in the number of stillborn offspring. However, the increased mortality of this group in the first week of life makes it seem very probable that an intoxication of a more severe degree would result in stillbirths among the offspring of lead fathers. When the mother is lead poisoned, stillbirths are numerous. The following table of the offspring of Lead Female No. 25 will illustrate this point:

TABLE II.
Offspring of Lead Female No. 25.

By free male No. 10	{	40 grams. Stillborn.
		18 " "
By free male No. 10	{	107 grams. Living. (Lead was withheld from the mother for 10 weeks, but was again administered during the last 3 weeks of pregnancy.)
By free male No. 10	{	54 grams. Stillborn.
		45 " "
		41 " "

The stillborn offspring of a lead mother are usually premature and often show by maceration of the skin that they have been dead for some time before expulsion.

Next to reduced birth weight, the high mortality during the first few days after birth was the most striking change shown by the offspring of lead males. As already noted in Table 3, from a total of one hundred and seventy pigs born in the course of this experiment eleven died during the first week after birth. Of these eleven, nine are found in the group of sixty-five offspring of lead males. While it is true that these guinea-pigs were underweight at birth, they were not premature but were well proportioned, had their eyes open and were quite active. Nor was their small size at birth the sole determining factor in causing their early death, for we have grown to maturity many of these dwarfed pigs weighing, for instance, 43, 44.5, 46 (3), 47, 48, and 49 grams at birth. Rather must it be considered that the small size at birth and the lessened vitality of the offspring of lead males are both manifestations of an injury to the male germ plasm. When both of these factors are present in a well-marked degree the offspring of such parents usually die in the first week.

In only two instances were malformations observed in the first filial generation. Two pigs showed a slight malformation of the upper jaw with deformed teeth. Occurring in so few instances, this malformation may have been due to some nutritional disturbance, and not to blastophthoria. However, special attention will be paid to the appearance of this or any other malformation in the second filial generation, upon which report cannot as yet be made.

A recovery of the germ plasm following the toxic injury is indicated by some of our results. On the charts showing the birth weights of all of the progeny of Lead Males Nos. 11 and 26 (Figs. 4 and 3) it will be noted that there is some tendency for offspring of more nearly average birth weight to be produced shortly after the intermissions in the administration of lead. Also in the case of Lead Female No. 25 (Table 11) the only living offspring, an active pig of more

than average weight at birth, was conceived during a period when the administration of lead was interrupted. If these are in reality examples of an apparent recovery of the germ plasm following the withdrawal of the injurious agent it seems probable that the blastophthoric effect is exercised upon that portion of the germ plasm which is in process of maturation and not upon the reservoir of germ plasm in the germinal epithelium. This point will require further investigation.

CONCLUSIONS. — From the entire series of matings the following conclusions can be drawn:

1. In chronic lead poisoning in guinea-pigs there is a definite blastophthoric effect. This can best be demonstrated upon the male germ plasm, in which case the blastophthoria manifests itself in some instances by sterility without loss of sexual activity, by a reduction of approximately twenty per cent in the average birth weight, by an increased number of deaths in the first week of life, and by a general retardation in development such that the offspring of a lead-poisoned male remain permanently underweight.

2. The offspring of a lead-poisoned female are underweight at birth and are very frequently stillborn. The number of stillbirths is out of proportion to the degree of intoxication of the mother and points either to a special susceptibility of the embryonic tissues to lead or to a blastophthoric effect upon the female germ plasm.

3. From the apparent recovery of reproductive power some time after stopping the administration of lead it seems that the deleterious effect must be suffered especially by that portion of the germ plasm which is undergoing maturation and not by that which is stored as undeveloped germinal epithelium.

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A DIRECT READING POTENTIOMETER FOR MEASURING AND
RECORDING BOTH THE ACTUAL AND THE TOTAL
REACTION OF SOLUTIONS.*

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In connection with some experiments on the biological effects of ultra-violet light, the writer found it necessary to make a large number of determinations of the reaction of albumen and other proteins. The conditions of the experiments were such that neither "buffer solutions" nor indicators could be used. It was decided, therefore, to make the determinations by measuring the potential of the hydrogen electrode. At first the usual apparatus for such measurements was set up. Then, in order to obtain more desk room, it seemed advisable to eliminate as far as possible all loose wiring, and to permanently mount the various parts of the apparatus upon a single base. It then appeared that much time could be saved if the apparatus were made to read in terms of reaction, rather than in terms of potential. Several possible arrangements were tried out, and finally the instrument to be described below was constructed. The instrument measures and records both the actual (effective) and the total (titratable) reactions of solutions, and plots a curve of the changes of actual reaction, as amounts of acids or bases are added to the solution.

The determinations can be made quickly, and with much greater precision than with indicators. The results are free from the subjective errors of judgment of color, and are obtained in the form of a printed record of both the actual and the total reaction of the solution. Such printed records become a valuable part of one's note-book. Moreover, the determinations can be made of the reactions of solutions, which, because of their own natural color, make it impossible to use indicators, or of solutions which, because of their peculiar chemical constitution, make the indicator method

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unreliable. Both of these conditions are very frequently met by the biologist. Many of his solutions are colored or cloudy, and their chemical composition is such that the indicators do not give sharp end points or give end points which are false. The pitfalls of the indicator methods of determining the actual and the total reaction of solutions have been pointed out by a number of recent writers, and need not be discussed here.

The determinations of the reactions of solutions by the electrical method is by no means new. But the instrument described below so increases the ease and the speed with which such determinations can be made that a description seems worth while. The physical-chemical basis of the method will be found in any text-book of physical chemistry, and the biological aspects are described by Michaelis,¹ Hildebrand,² and Clark.³

A very brief statement concerning the nature of acids and bases will be given, in order to indicate the point of view and to explain the method of reading the printed records. Pure distilled water dissociates into hydrogen and hydroxyl-ions. The extent of this dissociation is such that in one liter of water at 22° C. there is approximately $\frac{1}{10,000,000}$ of a gram of hydrogen-ions. That is, concentration of the hydrogen-ions is $\frac{1}{10,000,000}$ normal. (Atomic weight of hydrogen taken as 1.) (The numbers with which we have to deal in this paper contain so many figures that a shorter method of representing them will be adopted, namely: the multiples of some power of ten, for example, the fractions written above will be written 1×10^{-7} , and $\frac{3}{10,000,000}$ will be written 3×10^{-7} .) As there is one hydroxyl-ion formed for each hydrogen-ion, the concentration of the hydroxyl-ions must also be equal to 1×10^{-7} . According to the Mass Law, the concentration of the hydrogen-ions (H'_c) multiplied by the concentration of the hydroxyl-ions (OH'_c) equals the concentration of the water (H_2O_c) multiplied by a constant (K). The dissociation of the water is so slight that the concentration of the water may be considered as unity, and need not

appear in the equation. We may write $H_c \times OH_c = K$. The value of K at 22° C. is nearly 1×10^{-14} . In distilled water or water containing small amounts of neutral salts, the concentration of these two ions are equal. When acids or acid salts are added to the water, there is an increase in the number

Corrections for:—

“A DIRECT READING POTENTIOMETER FOR MEASURING AND RECORDING BOTH THE ACTUAL AND THE TOTAL REACTION OF SOLUTIONS,” BY

W. T. BOVIE.

- Page 296. 22d line from top, insert “the” after “that is.”
- Page 296. 3d line from bottom, cross out capital “K” and substitute small “k.”
- Page 296. Last line, cross out all of the sentence after “as” and substitute “constant.”
- Page 297. At end of first line, insert “($k \times H_2O_c = K$).”
- Page 302. 3d line, place period after “seconds;” “without disconnecting the battery” is to be read after “both electrodes” in the 4th line.
- Page 312. 8th line, change “nine-tenths” to “four-tenths.”
- Page 314. 13th line, cross out “and one-half” at the end of the line.

solution, the reaction of which is neutral, contains 1×10^{-7} grams of hydrogen-ions per liter, and a solution, the reaction of which is normal alkaline, contains 1×10^{-14} grams of hydrogen-ions per liter. The actual reaction of a solution differs from the total reaction, in that the total reaction takes account of all of the acid or alkali present, both the dissociated and the un-dissociated.

unreliable. Both of these conditions are very frequently met by the biologist. Many of his solutions are colored or cloudy, and their chemical composition is such that the indicators do not give sharp end points or give end points which are false. The use of the indicator methods of determining the

3 x 10⁻⁷.) As there is one hydroxyl-ion formed for each hydrogen-ion, the concentration of the hydroxyl-ions must also be equal to 1 x 10⁻⁷. According to the Mass Law, the concentration of the hydrogen-ions (H_c) multiplied by the concentration of the hydroxyl-ions (OH_c) equals the concentration of the water (H₂O_c) multiplied by a constant (K). The dissociation of the water is so slight that the concentration of the water may be considered as unity, and need not

appear in the equation. We may write $H'_c \times OH'_c = K$. The value of K at 22° C. is nearly 1×10^{-14} . In distilled water or water containing small amounts of neutral salts, the concentration of these two ions are equal. When acids or acid salts are added to the water, there is an increase in the number of hydrogen-ions, and since the Mass Law holds under this condition there must be a proportional decrease in the number of hydroxyl-ions. The reverse is true when alkalis or alkaline salts are added to the water. These relations may

be stated as follows: If $\frac{H'_c}{OH'_c} = 1$ the reaction of the solution

is neutral. If $\frac{H'_c}{OH'_c} > 1$ the reaction is acid, and if $\frac{H'_c}{OH'_c} < 1$ the reaction is alkaline. It will be seen, therefore, that no acid is free from hydroxyl-ions and no alkali free from hydrogen-ions, and furthermore, that the change from an acid to an alkaline reaction is not connected with a sudden change in the nature of the solution. If one knows the concentration of the hydrogen-ions the concentration of the hydroxyl-ions may be easily determined; therefore, the actual reaction of any solution, either acid or alkaline, may be indicated by giving the concentration of the hydrogen-ions. Since this makes it possible to define the actual reactions of all solutions, both acid and alkaline, in the units of a single scale of numbers, this method will be followed.

The actual reaction of all solutions will be given in terms of the concentration of the hydrogen-ions; and these concentrations will be expressed in the usual terms of normal solutions. A solution, the actual reaction of which is normal, contains one gram of hydrogen-ions per liter, and a solution, the reaction of which is neutral, contains 1×10^{-7} grams of hydrogen-ions per liter, and a solution, the reaction of which is normal alkaline, contains 1×10^{-14} grams of hydrogen-ions per liter. The actual reaction of a solution differs from the total reaction, in that the total reaction takes account of all of the acid or alkali present, both the dissociated and the un-dissociated.

Determinations of the actual reaction and of the manner in which this reaction varies with different conditions are usually of more value to the biologist than are determinations of the total reaction. The total reaction, however, is more frequently determined because of the time and the labor required by the present methods of determining actual reactions. It is hoped that the instrument described in this paper will increase the ease of determining both the actual and the total reactions of solutions and also of determining the manner in which the actual reaction of solutions varies with varying conditions.

The details of the electrical arrangements of this instrument will be described in a later paper, in general they are similar to those described by Hildebrand (l.c.), except that the quadrant electrometer is used in the place of the capillary electrometer or the galvanometer. The poles of the battery A (Fig. 1) are connected through a series of three resistances, B, C, and D. B and D are variable, C is a straight wire or an evenly spaced spiral. Two shunt circuits E and F in parallel connect the positive pole of the battery with the sliding contact G, which makes connections with the resistance C. One of the shunt circuits E includes a volt meter V, reading by one-hundredths of a volt up to 1.2 volts. The other shunt circuit F includes the calomel electrode H, the solution to be measured in the beaker L, the hydrogen electrode I, and the quadrant electrometer K. The quadrant electrometer can be cut out through the circuit N.

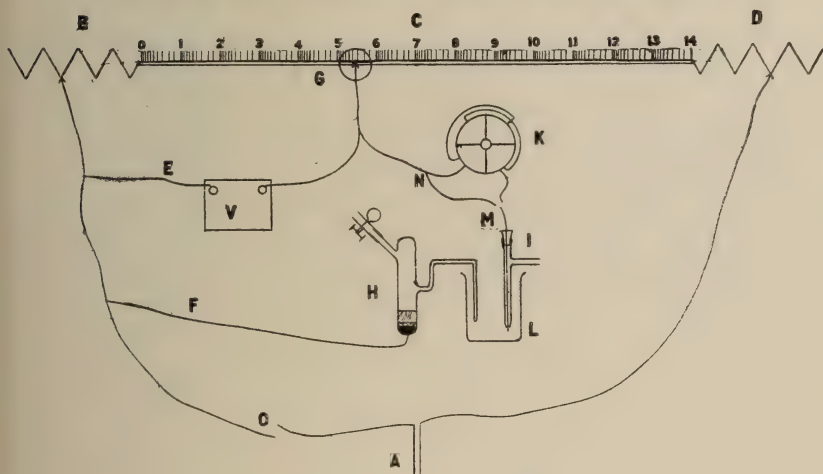
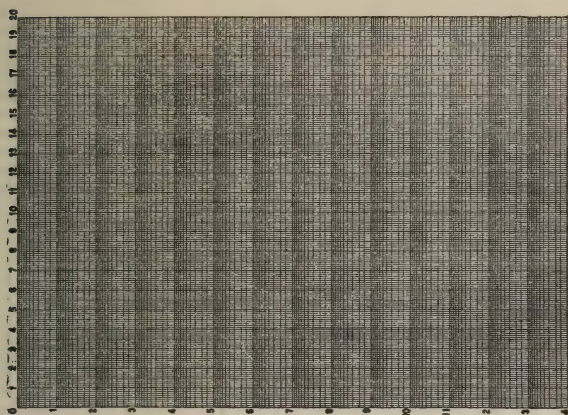


FIG. 1.

General directions for making the calomel and the hydrogen electrodes may be found in any text-book of physical chemistry. They may be made in a variety of forms. The writer has found the electrodes described here particularly convenient. They have been designed in such a manner that the beaker L (Fig. 1) may be replaced by either a culture tube or a flask. The calomel electrode is made up as follows: Three to four cubic centimeters of clean mercury, and five to ten grams of calomel are washed with several

changes of a saturated solution of potassium chloride (prepared by dissolving the salt in boiling water, cooling to room temperature, and separating out the excess of potassium chloride crystals) and placed in a large culture tube A (Fig. 2) twenty-five to thirty millimeters in diameter. The tube is then filled with saturated potassium chloride solution, and closed tightly by a two-hole rubber stopper. Contact is made with the mercury at the bottom of the tube by a platinum wire sealed through a glass tube B. C is a capillary glass tube filled with saturated potassium chloride solution, and connected through a T tube to the rubber tube D. The upper end of D is connected with a reservoir of saturated potassium chloride solution. The lower end connects through an ungreased glass stop-cock with the capillary tube E which dips into the beaker L (Fig. 1). The rubber tube D and capillary E are filled with a saturated potassium chloride solution, care being taken to exclude all air bubbles. The glass stop-cock is kept closed except when it is necessary to clean the electrode. It is then opened and enough potassium chloride solution run through E to wash out any solution which may have diffused up into it during previous determinations.

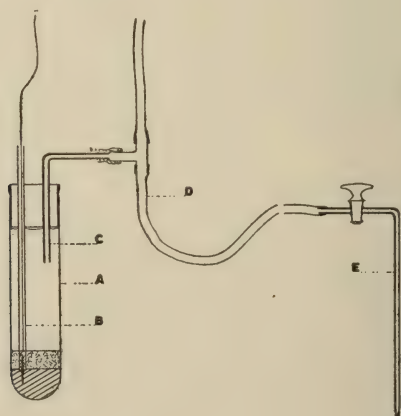


FIG. 2.

In all of the determinations which the writer has made it has been possible to use the hydrogen electrode with a stream of hydrogen gas. The electrode is very similar to the one described by Hildebrand (*l.c.*) except that the sheet of platinum has been replaced by a platinum wire. The outer tube A (Fig. 3) is six millimeters outside diameter, and fifteen centimeters long. The side arm connects with a hydrogen generator. The stream of gas is regulated by the "capillary" stop cock to one or two bubbles per second, or the gas may be permitted to flow faster, and thus effectively stir the solution in the beaker. The platinum wire is sealed through the small glass tube B which is two or three millimeters in diameter. Electrical connections are made through a drop of mercury contained within the tube.

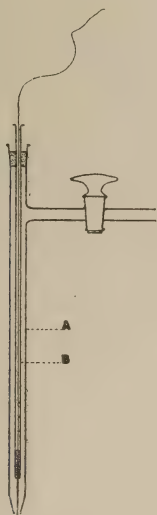


FIG. 3.

The platinum electrode is covered with platinum black by removing the tube B, connecting it with the negative pole of a battery consisting of two dry cells in series, and dipping it into a solution consisting of one gram of platinum chloride and ten milligrams of lead acetate in thirty cubic centimeters of distilled water. The circuit is completed by connecting a

second platinum electrode to the positive pole of the battery, and dipping it also into the platinum chloride solution. The electrode will be "platinized" in about thirty seconds without disconnecting the battery. Both electrodes are dipped into dilute sulphuric acid for one or two minutes. The electrode is now disconnected from the battery and rinsed with distilled water. It is advisable to keep several platinum electrodes platinized, ready for use. They must be kept in distilled water. If they are inadvertently permitted to become dry the platinum coat should be wiped off with a clean cloth, and the platinizing repeated. After fifteen or twenty determinations the electrode should be replatinized.

Associated with the resistance C (Fig. 1) is a scale over which the contact G plays. Upon this scale the hydrogen-ion concentration (actual reaction) of the solution is indicated. When the battery circuit is closed by the key O, current flows through the resistances B, C, and D, and we have a fall of potential from D to B which, when new dry cells are used for the battery at A, is equal to about 1.5 volts. The total resistance of B, C, and D should be great enough to avoid taking more than a few hundredths of an ampere from the battery. The solution to be tested is placed in the beaker L, which also contains the calomel and the hydrogen electrodes. The beaker with the electrodes and the solution to be tested form a battery. The potential developed by this battery depends upon the hydrogen-ion concentration of the solution which forms the electrolyte. It is our purpose to measure this potential and thus determine the reaction of the solution. The potential is measured in the following manner: when the key M is closed and the quadrant electrometer is thus short circuited, both pairs of its quadrants take the same potential, and the needle of the electrometer comes to a position of rest. The electrometer is set up in such a manner that a spot of light is reflected from the mirror on the needle to a scale on the wall. The position of this spot of light when the needle is at rest is noted (zero position). The key M is opened and the pair of quadrants thus connected with the hydrogen

electrode become charged to a potential, dependent upon the hydrogen-ion concentration of the solution in the beaker. The needle of the electrometer then turns to a new position of rest. Now since we have a drop in potential along the wire C, it is possible, if B and D be properly adjusted, to find a point on this wire to which we can move the contact G and again bring both pairs of quadrants of the electrometer to the same potential and when this point has been found and the needle of the electrometer has been brought to its zero position, one can read the potential of this point from the scale of the volt meter V, and by substituting this value into a mathematical equation, it is possible to calculate the reaction of the solution in the beaker L. If we have a suitable scale under the wire C, it is possible to read from this scale the reaction of the solution in L, and thus save ourselves the trouble of making a mathematical calculation for each reading of the volt meter. It is necessary, however, to adjust the resistances B and D so that the drop in potential along C covers the proper range of voltage. This is accomplished as follows: The calomel electrode is tested by placing a few cubic centimeters of "standard acetate mixture" (N. NaOH — 50 cubic centimeters, N. acetic acid — 100 cubic centimeters, distilled water — 350 cubic centimeters) in the beaker L, setting the electrodes in place, and measuring the potential developed. If the calomel electrode has been properly made the potential as read from the volt meter should agree to within two or three millivolts with the figures shown in column 3, Table 1. The battery circuit is now closed and the pointer G set at the left hand end of the scale, the resistance B is adjusted until the volt meter indicates the voltage as represented in column 1, Table 1, which has been compiled from Michaelis (l.c.) and refers to a calomel electrode which is made up with a saturated KCl solution, as described above. The pointer G is now set to thirteen on the scale and the resistance D is adjusted until the volt meter has the reading as shown in column 2, Table 1.

TABLE I.
Volt meter readings.

Temp. C.	1×10^0	1×10^{-13}	Standard Acetate Mixture.
18°	0.250 volts.	1.000 volts.	0.517 volts.
19°	0.250 "	1.002 "	0.518 "
20°	0.249 "	1.004 "	0.518 "
21°	0.248 "	1.006 "	0.518 "
22°	0.248 "	1.008 "	0.518 "
23°	0.247 "	1.010 "	0.519 "
24°	0.246 "	1.012 "	0.519 "
25°	0.246 "	1.014 "	0.519 "

The pointer is then moved back to the left hand end of the scale and the voltage is checked. (The figures given in columns 1 and 2 should be corrected by adding or subtracting the error in the calomel electrode as measured against the standard acetate mixture.) When the voltages of these two points on the scale have been made to correspond with the voltages as indicated in the table, the scale will read hydrogen-ions directly, in terms of normal solutions. This scale has a multiple logarithmic ruling of fourteen units. The numbers are the negative exponents of ten (the negative sign is omitted in the scale), hence the scale covers a range of hydrogen-ion concentrations from normal to 1×10^{-14} normal, that is from normal acid to normal base. (If these numbers are read as positive numbers they correspond to the PH numbers of Sörenson. It is better, however, to read them as negative exponents of ten, since this avoids the introduction of a new system of units for indicating reaction. If read thus, the numbers decrease as the hydrogen-ion concentration decreases.)

The space between any two numbers is ruled logarithmically, like the rulings of the slide rule, so that the whole

scale is as if fourteen slide rules were placed end to end. The values to be assigned to these logarithmic rulings will be understood from Figure 4. A few examples will make the method of reading clear. A reading at A is read 2×10^{-6} , at B, 6×10^{-6} , and at C, 10×10^{-6} or 1×10^{-5} , and at D, 4.5×10^{-6} . The contact G in Figure 1 points to 3.4×10^{-6} . It will be seen that the reading is to be associated each time with the negative exponent of ten to the right of it. A somewhat awkward but easily understood method of explaining the scale is as follows: the logarithmic reading is the numerator, while the number to the right of the reading indicates the number of zeros to be written after one in the denominator of a common fraction which represents the reaction of the solution in the usual terms of normality. 3.4×10^{-6} equals $\frac{3.4}{1,000,000}$ normal. If we wish to avoid the decimal point in the numerator we may write 34×10^{-7} or $\frac{34}{10,000,000}$ that is, the hydrogen-ion concentration is thirty-four times as great as that of distilled water.

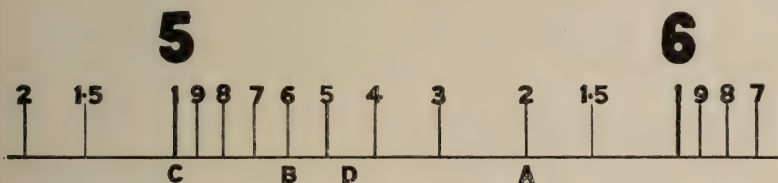


FIG. 4.

In the upper part of Figure 1 is shown a sheet of semi-multiple logarithmic coördinate paper. The ordinate ruling of this paper is like that of the scale described above. The abscissa ruling is linear, like that of ordinary cross-section paper, centimeter units divided into tenths. The ordinate units are negative, the abscissa units positive, and therefore the rulings lie in the lower right hand quadrant of the system of coördinates, and the origin is at the upper left hand corner of the sheet. We are more accustomed to plotting curves with both ordinate and abscissa values positive and at

first this paper may seem bottom side up, but this difficulty disappears as soon as one has used the paper a few times.

Placing the origin at the top has the advantage that a movement down the ordinate axis represents a decrease in the concentration of the hydrogen-ions. Curves representing the changes in hydrogen-ion concentration with the addition of acids or alkalies, or with other varying conditions such as time, growth of organisms, etc., are made by plotting the hydrogen-ion concentration as ordinates and the other variable as abscissæ. The instrument described in this paper is so arranged that the plotting is done automatically as follows: the cross-section paper is held by suitable clamps on a platen which can be moved back and forth under the resistance C. The platen moves in guides which hold it so that the ordinate ruling of the paper always registers with the scale from which the hydrogen-ion concentration is read. The moving contact G carries a printing mechanism, which, when pressed down, plots the hydrogen-ion concentration upon the cross-section paper. If the platen be moved to correspond with the burette readings in a titration or total reaction determination or with any other variable under investigation, it is evident that the desired curve will be generated. The curves illustrated in this paper have all been produced in this manner.

The method of determining the total reaction differs from the usual indicator method only in the fact that in this method we titrate to a particular hydrogen-ion concentration, while with the indicator method we titrate to the hydrogen-ion concentration at which the indicator changes color. In either method it is necessary to know the hydrogen-ion concentration to which one must titrate in order to have the acid and alkali present in equivalent amounts (equivalent point).

Obviously this is the actual reaction of the salt formed by the titration. This reaction may be determined by measuring the reaction of a solution of some of the pure salt, made up to an equal concentration. There are, however, other methods of determining the equivalent point.

Bjerrum⁴ gives methods of calculating the equivalent point from the dissociation constants of the acid and alkali used in the titration. According to Hildebrand (l.c.) the equivalent point is half way between the two inflection points of the titration curve.

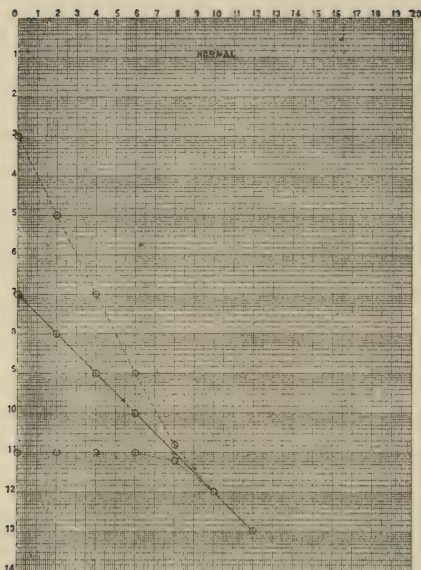
Bjerrum gives a table by the use of which if the dissociation constant of the acid is known, it is possible to determine the hydrogen-ion concentration of the alkali salt formed in the titration and also the hydrogen-ion concentration to which one must titrate in order to have the results accurate to within one per mille. The figures given in Bjerrum's table are the "PH" numbers of Sörenson. These figures have been translated into hydrogen-ion concentrations in the table (No. 2) given below. The first column (K_A) gives the dissociation constant of the acid. The succeeding columns (C_H and C_H interval) give the correct end-point reaction, and the reaction interval for one per mille accuracy for normal, tenth normal, hundredth normal, and thousandth normal salt formed by the titration. Blank spaces in the reaction-interval columns mean that the interval is too short to be measured by electrometric methods.

TABLE 2.
The correct end point reaction, and the reaction interval for the titration of an acid to an accuracy of 1 per mille.

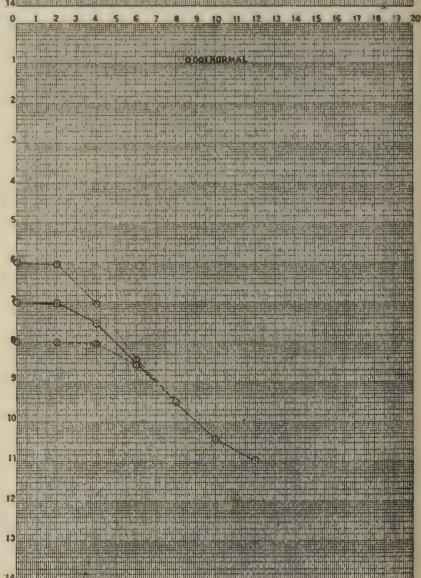
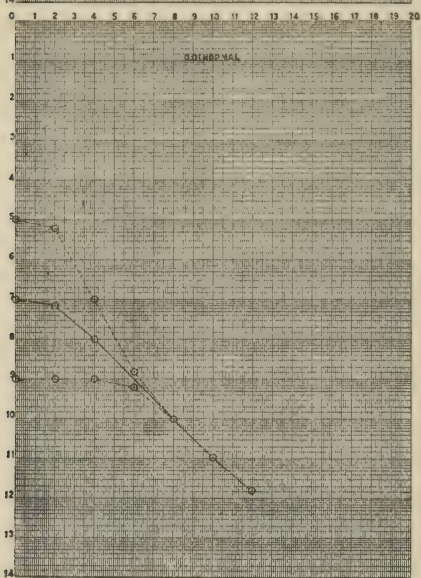
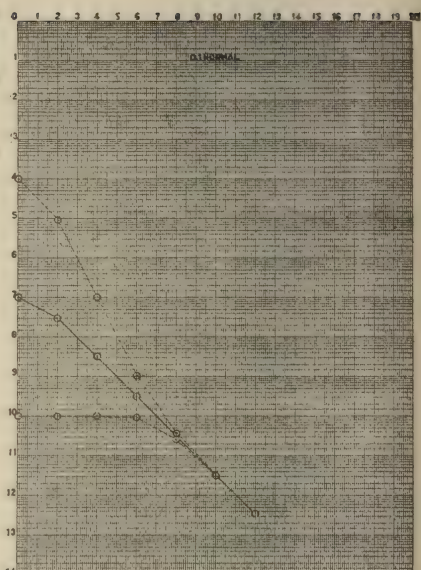
K_A	Titration to Normal Salt.		Titration to .1 Normal Salt.		Titration to .01 Normal Salt.		Titration to .001 Normal Salt.	
	C_H	C_H Interval.	C_H	C_H Interval.	C_H	C_H Interval.	C_H	C_H Interval.
∞	$1. \times 10^{-7}$	$1. \times 10^{-3}-1. \times 10^{-11}$	$1. \times 10^{-7}$	$1. \times 10^{-1}-1. \times 10^{-10}$	$1. \times 10^{-7}$	$1. \times 10^{-5}-1. \times 10^{-9}$	$1. \times 10^{-7}$	$1. \times 10^{-6}-1. \times 10^{-4}$
10^{-2} . . .	$1. \times 10^{-8}$	$1. \times 10^{-5}-1. \times 10^{-11}$	$3. \times 10^{-8}$	$9.1 \times 10^{-6}-1. \times 10^{-10}$	7.1×10^{-8}	$7.2 \times 10^{-6}-1. \times 10^{-9}$	9.5×10^{-8}	$9.1 \times 10^{-7}-1. \times 10^{-5}$
10^{-4} . . .	$1. \times 10^{-9}$	$1. \times 10^{-7}-1. \times 10^{-11}$	3.2×10^{-9}	$1. \times 10^{-7}-1. \times 10^{-10}$	$1. \times 10^{-8}$	$1. \times 10^{-7}-1. \times 10^{-9}$	$3. \times 10^{-8}$	$9.1 \times 10^{-8}-9.1 \times 10^{-9}$
10^{-6} . . .	$1. \times 10^{-10}$	$1. \times 10^{-9}-1. \times 10^{-11}$	3.2×10^{-10}	$1.1 \times 10^{-9}-9.1 \times 10^{-11}$	$1. \times 10^{-9}$	$1.6 \times 10^{-9}-6.3 \times 10^{-10}$	3.2×10^{-9}	$3.7 \times 10^{-9}-2.7 \times 10^{-7}$
10^{-8} . . .	$1. \times 10^{-11}$	$1.6 \times 10^{-11}-6.2 \times 10^{-12}$	3.2×10^{-11}	$3.7 \times 10^{-11}-2.6 \times 10^{-11}$	$1. \times 10^{-10}$	$1.05 \times 10^{-10}-9.5 \times 10^{-11}$	3.2×10^{-10}
10^{-10} . . .	$1. \times 10^{-12}$	$1.05 \times 10^{-12}-9.5 \times 10^{-13}$	3.2×10^{-12}	1.05×10^{-11}	3.7×10^{-11}
10^{-12} . . .	1.05×10^{-13}	3.4×10^{-13}	1.6×10^{-12}	1.1×10^{-11}

For convenience in interpolation the data given in Table 2 have been represented graphically in Curves 1, 2, 3, and 4. In these curves the ordinate values are hydrogen-ion concentrations, the abscissa values are the negative exponents of the acid dissociation constants (*e.g.*, the dissociation constant 10^{-4} is plotted four centimeters from the ordinate axis). The continuous line indicates the reaction of the correct end point. The broken lines mark the reaction interval for one per mille accuracy.

CURVE No. 1.



CURVE No. 2.



CURVE No. 3.

CURVE No. 4.

The titration curves of many physiological solutions, *e.g.*, culture media, do not have clearly defined end points, and it becomes necessary to select arbitrarily an end point at a hydrogen-ion concentration depending upon the nature of the determinations which are being made. Under these conditions the electrical method of titration is much more convenient than the indicator method, for instead of being obliged to titrate to the end point of some indicator one can titrate to any desired hydrogen-ion concentration.

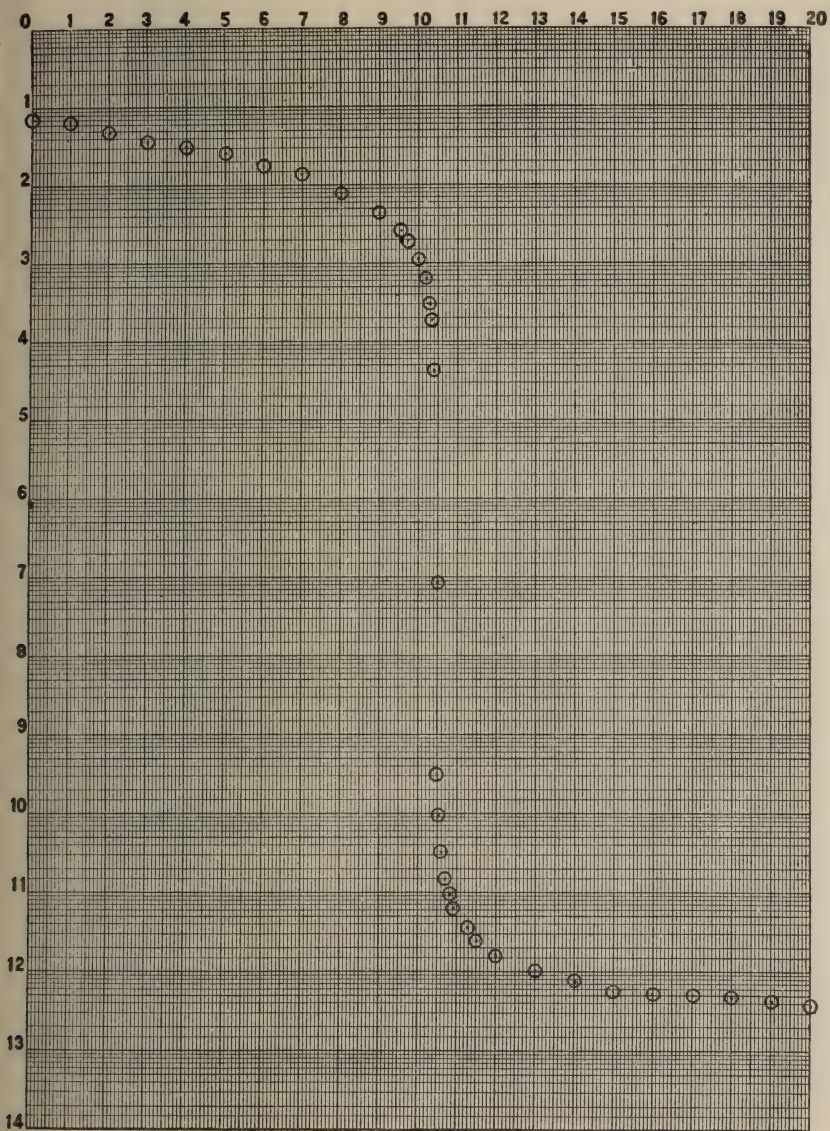
In regard to the accuracy of the method as compared with the indicator method: According to Sörenson the turning point of a good indicator under the best of conditions covers a range of from one to two of the larger units, *i.e.*, one to two-fourteenths of the entire scale described above. Bjerrum points out that these figures apply only to the range covered by the sharpest color change, the entire change covering a range from two to three units of the scale. By ordinary methods an expert with a good indicator can determine the hydrogen-ion concentration to about .3 unit. With the instrument described above one can measure the voltage to an accuracy of one millivolt; this means a determination of the hydrogen-ion concentration to an accuracy of .017 of one of the units on the scale.

CURVE NO. 5.

The Titration of Ten Cubic Centimeters of H_2SO_4 with .1 Normal NaOH.

One centimeter on the abscissa axis represents one cubic centimeter of NaOH.

As the NaOH is added to the solution the hydrogen-ion concentration falls gradually until nine cubic centimeters have been added. The further addition of alkali causes the hydrogen-ion concentration to decrease more rapidly. After ten and nine-tenths cubic centimeters have been added the curve falls precipitously from a hydrogen-ion concentration of 4.3×10^{-5} to 3.1×10^{-10} . Further addition of alkali produces a relatively small change in the hydrogen-ion concentration. For an accuracy of one per cent the end point in this titration may be taken at practically any point along the precipitous drop in the curve. The equivalent point is at 1×10^{-7} .



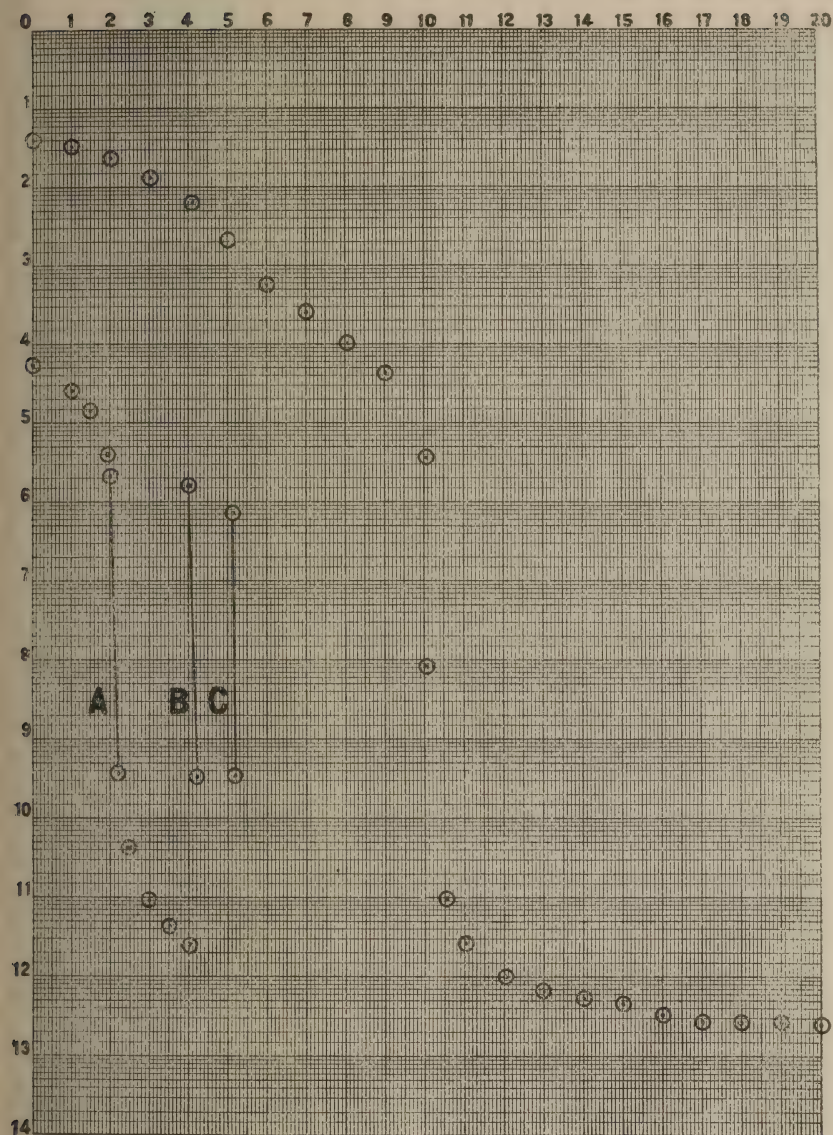
CURVE No. 5.

CURVE NO. 6.

The Titration of Ten Cubic Centimeters of Oxalic Acid with .1 Normal NaOH.

Abscissa readings, one centimeter equals one cubic centimeter of alkali.

The addition of alkali causes the hydrogen-ion concentration to drop more rapidly in this titration than in the titration of the sulphuric acid, shown in Curve No. 5. It will be noted that there is a slight break in the curve between the abscissa readings 4 and 6. As pointed out by Hildebrande this break has to do with the fact that oxalic acid is a dibasic acid, and the dissociation constant of the two hydrogen-ions are sufficiently far apart to permit one to identify their individual influence upon the hydrogen-ion concentration. The precipitous drop in this curve occurs when ten and one-half cubic centimeters of alkali have been added. The equivalent point, if taken midway between the upper and lower inflection points of the curve, is at 8×10^{-9} . When the potentiometer is used for routine work it is not necessary to develop the entire curve. The procedure is similar to that adopted when indicators are used. One adds quickly amounts of alkali until the end point is nearly reached. This is determined by setting the sliding contact G of the potentiometer to the hydrogen-ion concentration of the equivalent point and then watching the spot of light from the electrometer. Just before reaching the end point, the burette readings are noted and finally the addition of a single drop takes us past the equivalent point. The curves marked A, B, and C were made in this manner. The abscissa readings of the coördinate paper do not apply to these curves. The two readings which are joined by the straight lines represent the change in hydrogen-ion concentration with the addition of a single drop from the alkali burette.



CURVE No. 6.

CURVE No. 7.

The Titration of Ten Cubic Centimeters of Ortho Phosphoric Acid with
.1 Normal NaOH.

Abscissa readings, one centimeter equals one cubic centimeter of alkali.

In this curve we see two precipitous drops, the first one occurring when the primary sodium phosphate is formed, and the second one with the formation of the secondary sodium phosphate. The tertiary sodium phosphate does not exist as such in solution, and hence does not appear in the curve.

CURVE No. 8.

The Titration of Ten Cubic Centimeters of a Nearly Saturated Solution of
Boracic Acid with Normal Sodium Hydroxide.

Abscissa readings, one centimeter equals one cubic centimeter of alkali.

The precipitous drop in this curve is very short and occurs, far to the alkaline side of the neutral point for distilled water, between 1×10^{-11} and 1×10^{-12} , and was reached when 6.9 cubic centimeters of normal alkali had been added. The dissociation constant of boracic acid is 1.1×10^{-9} . By reference to Table 2 and the interpolation curves, it will be seen that the equivalent point is about 3×10^{-12} and that the range for one per mille accuracy is from 2.5×10^{-12} to 4×10^{-12} . It would not be possible by indicator methods to make this titration to an accuracy of one per cent, even if an indicator with the proper end point could be found. The boracic acid solution was .69 normal. This curve brings out very forcibly the difference between actual and total reaction. The total reaction, .69 normal, is marked by the cross on the ordinate axis near the top of the sheet. The actual reaction is 6.5×10^{-5} . This is but little more acid than the average laboratory distilled water when it has absorbed its full quota of carbon dioxide from the air. A saturated solution of boracic acid, while having a low hydrogen-ion concentration, serves as a reservoir for a large amount of available hydrogen-ions. In this titration, with the first addition of alkali, there was a very rapid drop in the hydrogen-ion concentration. This is due to the fact that the alkali salt of the boracic acid is strongly hydrolyzed

and as a consequence the dissociation of the boracic acid is repressed.

CURVE NO. 9.

The Effect of a Suspension Colloid upon the Actual Reaction of a Solution.

Curve A is the titration of ten cubic centimeters of H_2SO_4 with .01 normal NaOH. Curve B is the titration of a second ten cubic centimeters of the acid to which has been added .5 gram of Kahlbaum's animal charcoal. The charcoal adsorbs both the hydrogen and hydroxyl-ions. The titration curve is straightened out and the sharp end point obliterated.

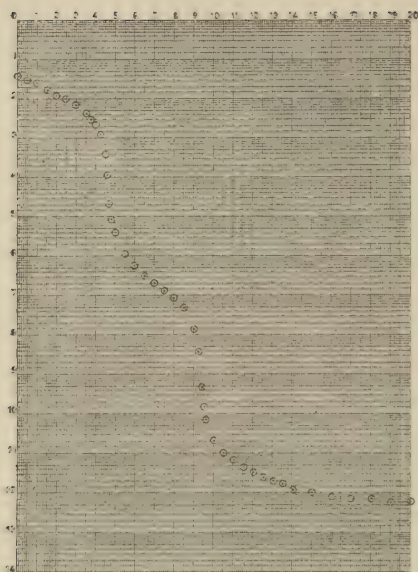
CURVE NO. 10.

The Titration of Ten Cubic Centimeters of Acetic Acid with .1 Normal NaOH.

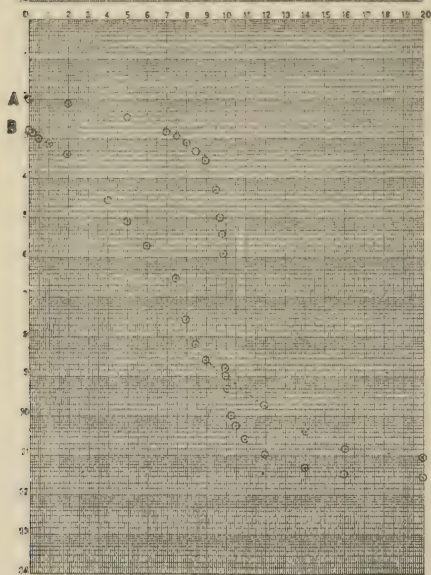
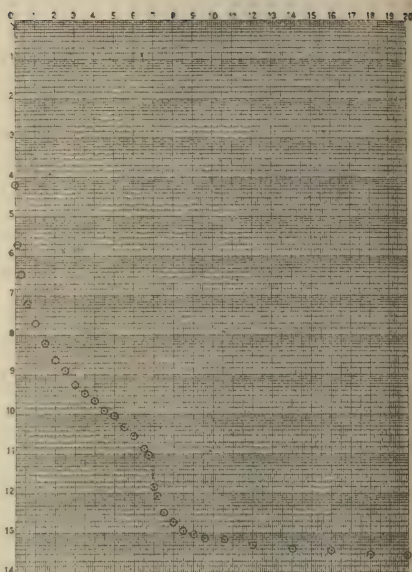
The abscissa readings, one centimeter equals one cubic centimeter of alkali.

Acetic acid is a weak acid (dissociation constant = 1.86×10^{-5}) and is slightly dissociated. Although the total reaction of the acid is over .1 normal the actual reaction is but .002 normal. The actual reaction decreases rapidly with the addition of the first alkali; due to the repression of the dissociation of the acid. In this case we are titrating a weak acid with a strong base. The sodium acetate which is formed is an alkaline salt, hence the equivalent point is on the alkaline side of the neutral point of distilled water. The concentration of the salt formed is .05 normal. From Curves 2 and 3 it will be seen that the equivalent point should be at 2×10^{-9} , and this point is half way between the two inflection points of the curve.

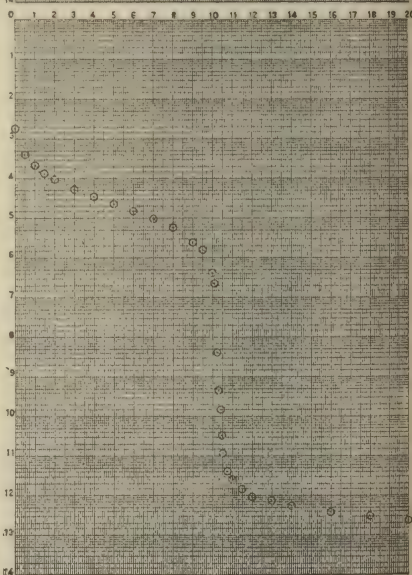
CURVE No. 7.



CURVE No. 8.



CURVE No. 9.



CURVE No. 10.

CURVE NO. II.

The Addition of Sodium Acetate to Acetic Acid and the Titration of the Mixture with .1 Normal NaOH, and .1 Normal H_2SO_4 .

Curve No. II is plotted on coördinate paper which has the origin placed so as to give both positive and negative values on the abscissa axis. The cross at 1×10^{-1} on the ordinate axis marks the total reaction of acetic acid as determined by titration with .1 normal NaOH. The actual reaction is plotted at 1.8×10^{-3} . To the ten cubic centimeters of acetic acid from which this reading was taken, sodium acetate was added, in various amounts and readings of the hydrogen-ion concentration taken after each addition. The amounts of sodium acetate are indicated by the negative abscissa readings, five centimeters representing an amount of sodium acetate sufficient to make a normal solution of the salt. The addition of sodium acetate represses the dissociation of the acid. After the sodium acetate had been added, the sheet was moved back and the resulting hydrogen-ion concentration plotted on the ordinate axis at A. The solution was then titrated with .1 normal sodium hydroxide. This titration is the left hand half of the lower curve. The additions of alkali are represented as negative abscissa readings, one centimeter equals two cubic centimeters. A similar solution was then made up of .1 normal acetic acid plus sufficient sodium acetate to form a normal solution of the salt, and the solution titrated with .1 normal sulphuric acid. This titration is represented by the right hand half of the lower curve. The additions of acid are represented as positive abscissa readings, one centimeter equals two cubic centimeters. Acetic acid plus sodium acetate is one of the regular "buffer" solutions. This "buffer" action is clearly shown by the curve. The addition of considerable amounts of either acid or alkali produces but slight change in the hydrogen-ion concentration. It will be noted that the alkali titration curve drops precipitously when a little over ten cubic centimeters have been added. The titration of this sodium acetate mixture gives us the total acid contents. A titration with an indicator such as phenolphthalein would not indicate the original actual reaction of the solution. The two

reactions are quite different, the total reaction being .1 normal, the actual reaction 4×10^{-6} normal, less acid than laboratory distilled water containing carbon dioxide.

CURVE No. 12.

The Titration of Ten Cubic Centimeters of HCl with Ammonia.

This is a strong acid with a weak base and the equivalent point of the titration is on the acid side of distilled water.

CURVE No. 13.

A Nitrogen Determination by Kober's Modification of the Kjeldahl Method.

The ammonia was collected in .02 normal HCl. The curve at the right is the titration of ten cubic centimeters of the original acid with sodium hydroxide. The curve at the left is the titration of ten cubic centimeters of the acid after collecting the ammonia. It is of interest to note the similarity in the shape of this curve and the shape of Curve No. 12. The titration was conducted with a stream of hydrogen gas and when sufficient alkali had been added to drop the hydrogen-ion concentration below 5×10^{-10} the dissociation of the ammonium hydroxide was repressed and the ammonia formed was washed out of the solution. The curve follows along the dotted line and finally merges with the curve of the titration of the HCl alone.

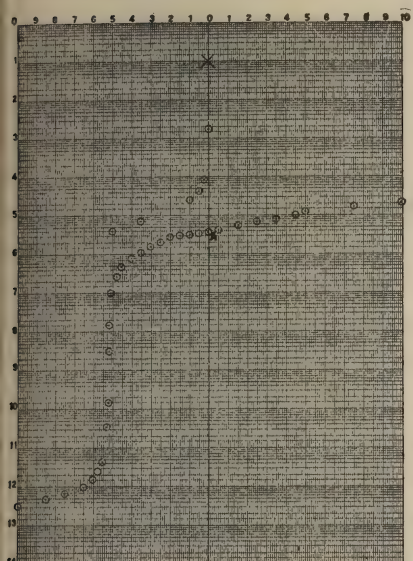
CURVE No. 14.

The Titration of Ten Cubic Centimeters of Bouillon with .05 Normal NaOH and .05 Normal H_2SO_4 .

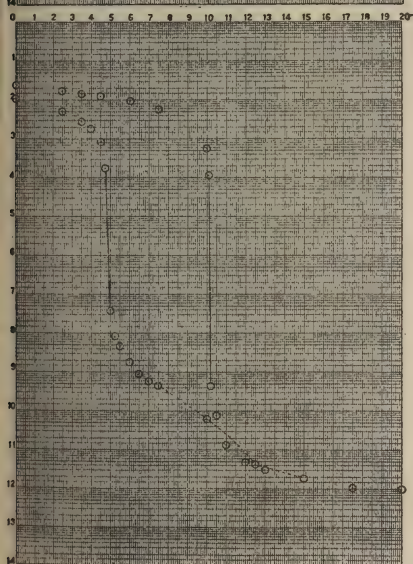
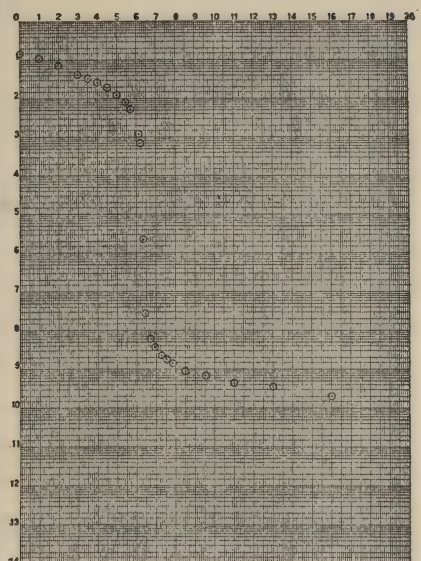
One centimeter equals one cubic centimeter of alkali and acid.

The reaction of this culture media had been "adjusted" and was found to be 5.4×10^{-8} . This reaction is plotted on the ordinate axis. The change of hydrogen-ion concentration produced by the addition of acid is shown in the right hand, and by the addition of alkali in the left hand, half of the curve. The ease with which culture media may be brought to any desired reaction is illustrated by this curve. One sets the contact G of the potentiometer to the desired reaction and then adds either acid or alkali until the electrometer needle comes to its zero position. It is not necessary to use standardized solutions.

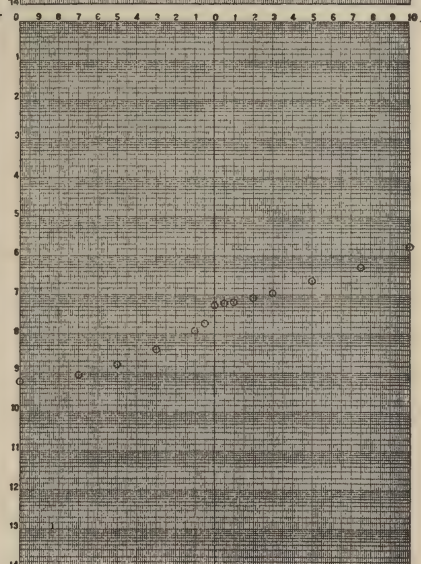
CURVE No. 11.



CURVE No. 12.



CURVE No. 13.



CURVE No. 14.

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T H E

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THE EFFECT OF ANTIPLATELET SERUM ON BLOOD PLATE-
LETS AND THE EXPERIMENTAL PRODUCTION OF PUR-
PURA HEMORRHAGICA.*

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The occurrence of a greatly diminished number of blood platelets in purpura hemorrhagica has been a well recognized fact for many years. Hayem¹ and Denys² first pointed out the important part played by the blood platelets in this form of purpura. More recently Duke³ has made excellent clinical studies of this condition.

With the establishment of the constant association of diminished blood platelets and purpura hemorrhagica, attention has been turned to the cause of this diminution of blood platelets and to experimental reproduction of this condition. The problem has been attacked from several different angles. Cole⁴ in 1907 produced an antiplatelet serum by the repeated injections of alien blood platelets. He observed marked agglutination of platelets with this serum in high dilutions. He also found that this serum was specific for the blood platelets of the species which furnished the platelets for immunization. Stchastnyi⁵ showed that during the process of platelet agglutination in vitro by an antiserum, complement is absorbed. However, he does not state that complement is necessary for the reaction to take

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place. LeSourd and Pagniez⁶ injected animals intravenously with antiplatelet serum and were able to produce a condition of the blood closely resembling that found in purpura hemorrhagica. There was a rapid disappearance of platelets, the blood clotted in normal time but the clot failed to retract. They had previously showed that retraction of the clot depended on the presence of an abundance of platelets. They found also that this serum was specific. These findings were confirmed by Achard and Aynaud.⁷

Ledingham⁸ studying the action of an antiserum to platelets both in vivo and in vitro directed his attention principally towards two points. He demonstrated, first, that the antiplatelet serum agglutinated platelets in vitro even in high dilutions, and, second, that the lesions produced by the injection of this serum in vivo were typical of those seen in purpura hemorrhagica in man. In a later communication Ledingham and Bedson⁹ noted the reduction in platelet count in the animals with experimental purpura hemorrhagica. Duke¹⁰ using large doses of benzol and sublethal doses of diphtheria toxin was able to produce a marked fall in the number of platelets. This effect was by no means a constant one and in only one case, which followed the injection of diphtheria toxin, did purpura hemorrhagica occur. The bone marrow in the rabbits treated with benzol was found to be completely aplastic. Similar observations of experimental reduction of platelet counts have been made by other investigators using a considerable variety of poisons. The diminution of platelets in most of these instances was incidental and the destructive agent was apparently not specific for the blood platelets.

The antiplatelet serum used in our experiments was produced by injecting a rabbit intravenously at weekly intervals with blood platelets obtained from forty cubic centimeters of guinea-pigs' blood. This dose was repeated four times, so that in all the animal received the platelets which we were able to isolate from about one hundred and sixty cubic centimeters of blood. The first serum was obtained from

the rabbit a week after the last injection. Subsequent bleedings were made whenever fresh serum was needed.

Platelets were obtained for the experiments as follows: Blood was taken rapidly from the hearts of guinea-pigs into a syringe containing oxalate. The oxalated blood was centrifugated at low speed until most of the platelets had been deposited in the "buffy coat," usually about ten minutes. This layer of platelets was pipetted off and washed with salt solution in a specially constructed centrifuge tube. Glass tubing one centimeter in diameter was drawn out at one end to a long fine point. The red cells which had been carried over with the buffy coat were deposited in the bottom of the tube and the narrow, relatively deep layer of platelets above could easily be taken off. After the second washing a suspension of the platelets could be obtained which was practically free from red blood corpuscles. The desirability of having a platelet emulsion as free as possible from red cells is due to the fact that an antiplatelet serum usually contains a hemolysin and a hemagglutinin since in the production of the serum it is difficult to obtain platelets in quantities sufficient for injection absolutely free from red blood corpuscles.

The platelets of the "buffy coat" were used in preference to those of the cloudy plasma for two reasons. In the first place a larger yield of platelets could be obtained and in the second place they were found to be considerably more sensitive to the action of the antiplatelet serum than were the platelets which remained suspended in the plasma. Isolated blood platelets in thick suspension were readily obtained by this method. The blood platelets obtained from the supernatant plasma, which were used by the other observers, tended in our experience to be spontaneously clumped. This clumping interferes markedly with the tests.

AGGLUTINATION AND LYTIC TESTS.

TABLE I.

Antiplatelet serum 24 hours old.

Dilutions.	Agglutination and Lysis.	
	Normal Rat Serum.	Antiplatelet Serum.
1:1.	+	+
1:2.	+	+
1:3.	+	+
1:4.	+	+
1:5.	+	+
1:6.	+	+
1:8.	O (partial in 2 hours).	+
1:10.	O (partial in 3 hours).	+
1:20.	O (slight in 3 hours).	+
1:40.	O (very slight in 3 hours).	+
1:80.	O	+
1:160.	O	± (almost complete in 3 hours).
1:320.	O	± (partial in 3 hours).

The serum was diluted with normal salt solution. The total volume used in each test was .5 cubic centimeter of the diluted serum. One drop of a moderately dense suspension of fresh blood platelets was added. The resulting mixture was definitely cloudy.

The readings were made at the end of an hour. The readings repeated at the end of twenty-four hours showed little further change. A test was called positive only when water-clear. In a one to three dilution of the normal serum it required ten minutes or more for lysis to occur and in a dilution of one to six a full hour had elapsed before the reaction was completed. In the dilutions of antiplatelet serum one to eighty, lysis occurred in ten minutes. When the reaction

occurred rapidly agglutination and lysis seemed to proceed simultaneously. As it became slower, however, agglutination was present for some time before lysis was apparent. In those tests marked "partial" this separation of the two actions was more definite, agglutination being definite but lysis slight or absent. The fact that agglutination occurred in higher dilutions than lysis suggests one of two possibilities: first, that the serum has two distinct actions, one an agglutinating and the other a lytic, and that the former is the stronger of the two; second, that the reaction is a single process beginning with agglutination and when the serum is present in sufficient concentration, going on to lysis. The specificity of this serum was next tested by determining its action on rabbit platelets. The resulting effect was no greater than that produced by normal serum.

We have observed the phenomena of agglutination and fusion of the blood platelets under the microscope. The contact with glass and drying intrude such disturbing factors that this method did not seem suitable in testing the activity of the various dilutions. Fresh sera from any species, active thrombin, and serozyme in the presence of calcium, will cause a striking agglutination and fusion of platelets in fifteen minutes to an hour. On the other hand, solutions of fibrinogen, oxalated plasma, and calcium alone cause no agglutination or fusion. In general this action of the blood platelets is a part of their function in the process of coagulation, in which they play an important part, and takes place with the other elements of coagulation from alien species as well as from the same species but only in the lowest dilutions. However, the action of the antiplatelet serum on blood platelets while apparently somewhat similar to the action of these other elements differs markedly. In the first place the action is specific for the blood platelets of one species. Secondly, the antiplatelet serum acts in very high dilutions. In the third place thrombin and serozyme are destroyed by heating to 56° C. for one hour. The activity of the antiplatelet serum is not destroyed by that amount of heat as

we show later. Therefore the specific action of antiplatelet serum on blood platelets is not identical with the similar action of normal serum on blood platelets.

This reaction requires the presence of complement. Table II. shows that inactivated antiplatelet serum by itself is without effect. When complement is added, however, lysis is just as marked as with fresh antiplatelet serum.

TABLE II.
Inactivated serum.

Dilutions.	Agglutination and Lysis.	
	Normal Rabbit Serum.	Antiplatelet Serum, Inactivated.
I : 10	O	O
I : 20	O	O
I : 40	O	O
I : 80	O	O
I : 160	O	O
I : 320	O	O

After adding .05 cubic centimeter guinea-pig serum to each tube as complement.

I : 10	O	+
I : 20	O	+
I : 40	O	+
I : 80	O	+
I : 160	O	±
I : 320	O	±

Furthermore, it was determined that complement is actually used up in this reaction since sensitized red cells failed to hemolyze when added to a mixture of inactivated antiplatelet serum, platelets, and complement which had previously been in contact for one hour.

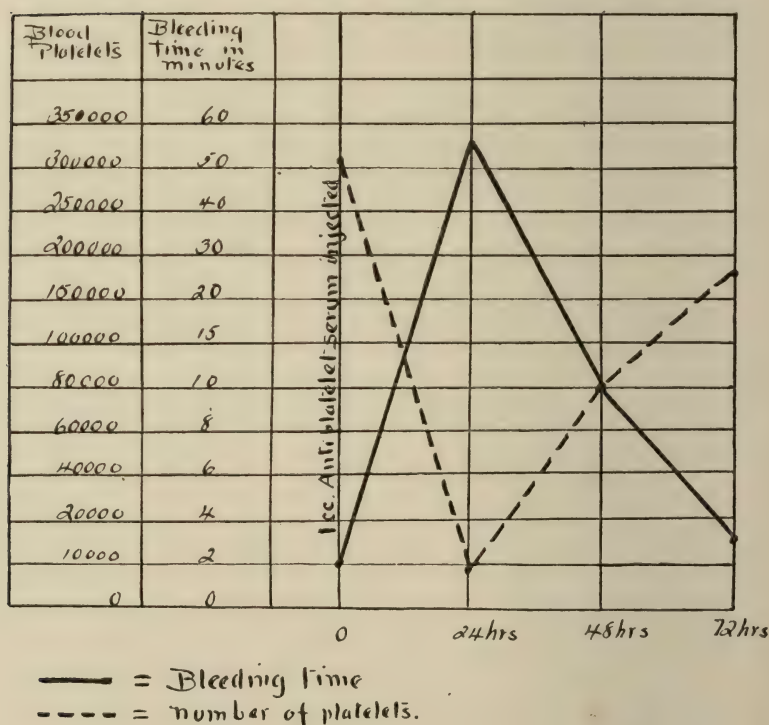
Action of antiplatelet serum on whole blood. — We also observed the effect of antiplatelet serum on the platelets in the presence of the other blood constituents. Nine cubic centimeters of blood were therefore drawn from the heart directly into a syringe containing one cubic centimeter of antiplatelet serum and one cubic centimeter of one per cent oxalate solution. This mixture was divided into two portions: One portion was centrifugated immediately. The yield of platelets was considerably diminished and the platelets were clumped. The other portion was kept on ice for twenty-four hours and then centrifugalized. The buffy coat was practically absent and the few platelets which could be obtained were collected in structureless clumps which had a fused vacuolated appearance. Nine cubic centimeters of blood were also taken into one cubic centimeter of antiplatelet serum. This mixture clotted in the usual time (four minutes). The clot, however, did not retract. (The retraction of the clot depends on the presence of an abundance of normal active platelets.)

Animal inoculation with antiplatelet serum. — Guinea-pigs weighing from three hundred to four hundred grams were inoculated in three ways — subcutaneously, intraperitoneally, and directly into the heart. Controls were injected in the same way with the same amount of normal rabbit serum.

Subcutaneous injection. — Three animals were given subcutaneous injections of one cubic centimeter each. All developed purpuric spots. The intensity and rapidity of onset of the symptoms produced varied considerably. One pig, No. 29, showed within twenty-four hours about the site of inoculation an area of induration one to two centimeters wide extending almost the entire length of the abdomen on that side. Over this area the skin was much reddened and showed many petechiæ which were also present on other parts of the abdomen. Blood was oozing from the rectum. The bleeding time and the platelet counts of this animal are shown in Table III. We utilized a suggestion of Duke's in regard to the estimation of the bleeding time. A

small vessel in the ear was pricked. The blood was gently sponged off every one to three minutes until the bleeding had ceased. Normally bleeding stops within five minutes. The platelet counts were made according to the method described by Wright and Kinnicutt.¹¹ We found platelet counts in normal guinea-pigs to vary from 200,000 to 550,000. On the third day the animal appeared practically normal; all the purpuric spots had disappeared.

Table III
Guinea pig No. 29.



In Pig No. 1 the effect was much more gradual, the reaction reaching its height on the sixth day after injection. Blood taken from the heart into oxalate on the seventh day yielded almost no platelets and a blood film made from the ear at this time showed only an occasional platelet. The

coagulation time was only three minutes by the method described by Lee and White.¹² The normal time was three to five minutes. The clot did not retract. Death from hemopericardium followed the bleeding.

Guinea-pig No. 7 died in seventy-two hours with fairly numerous subcutaneous hemorrhages.

Post-mortem findings in Pigs No. 1 and No. 7 were identical. On section the indurated area was found to consist of a mass of necrotic material among which were large hemorrhages and fibrin deposits. The spleen had a peculiar blackish appearance and the lungs showed frequent small infarcts.

Three guinea-pigs were injected with normal rabbit serum as controls. These animals showed no symptoms whatsoever. The bleeding time and platelet counts remained unchanged.

Intraperitoneal injection. — Six guinea-pigs were injected intraperitoneally, five receiving one cubic centimeter and one .5 cubic centimeter of antiplatelet serum. One cubic centimeter seemed to be about the lethal dose, as one pig died in thirty-six to forty-eight hours and two recovered after very severe reactions. The other two receiving one cubic centimeter of serum died after puncture of the heart to obtain blood for study.

These animals all behaved in practically the same way and all showed purpura during life. Twenty-four hours after injection the animal looked sick. Petechiæ were present on the abdomen. In one case there was a copious uterine hemorrhage. The normal bleeding time of two to five minutes was delayed to sixty to seventy-five minutes and the platelets were reduced to as low as 16,000. The coagulation time, taken in the three pigs which were bled, varied from five to eight minutes. The clots showed no retraction which corresponds to a diminished platelet count.

The animals which recovered showed the same constant relationship between the diminution of platelets and the prolonged bleeding time. Pig No. 20, which was typical of the series, showed twenty-four hours after injection a bleeding time of seventy-five minutes and a platelet count of 24,000. In forty-eight hours the bleeding time was five minutes and

the platelet count 220,000 and the pig was apparently perfectly well.

The number of platelets was also estimated in other ways. Blood taken from the heart into oxalate at the height of the reaction gave a very small yield of partially clumped platelets. A blood film from the ear made at the same time showed a marked diminution in the number of platelets; those present were all collected into large clumps, many of which were simply structureless masses of granular material.

The post-mortem findings were constant. They were similar but more marked than in the guinea-pigs inoculated subcutaneously. Small hemorrhages of varying size were present in abundance in the skin, abdominal muscles, intestines, the heart muscle, thoracic wall, and in the female in the uterus. The lungs showed multiple infarcts. The spleen was blackish red to black and the bone marrow darker red than normal.

Three control guinea-pigs inoculated with one cubic centimeter normal rabbit serum showed no change in the bleeding time or platelet count and exhibited none of the symptoms shown by the test animals.

Intracardiac injection. — Nine guinea-pigs received anti-platelet serum directly into the heart. The lethal dose appeared to be about .5 cubic centimeter, as all the pigs which were given a larger dose than this died. After 1.5 cubic centimeters death occurred within two to three minutes, after one cubic centimeter within two to five hours. Of the two pigs receiving .5 cubic centimeter one died at the end of twelve hours and the other showed a severe reaction but recovered in forty-eight hours.

During the first four or five minutes following the injection of one cubic centimeter of serum there was usually marked dyspnea and general malaise, which soon became less severe. The platelets showed a rapid decrease and the bleeding time soon became much delayed. Purpura was observed in no case either during life or at post-mortem. Pig No. 34, on which the platelet count and bleeding time

was done at frequent intervals, is a good example of this type of reaction. Before injection, the bleeding time was two minutes and the platelet count was 312,000. Fifteen minutes after injection the bleeding time was twenty minutes and the number of platelets had decreased to 72,000. At the end of thirty minutes the platelets had decreased to 12,000. One hour after injection the pig showed a bleeding time of forty-five minutes and a platelet count of 4,000. The animal died very soon afterwards.

The post-mortem findings varied with the length of time the animal lived after injection. The two pigs dying shortly after the injection of 1.5 cubic centimeters showed no signs of hemorrhage except for a few small infarcts in the lungs. There was, however, an intense congestion of all the viscera — especially of the spleen. Those which lived from periods of two to five hours showed multiple infarcts in the lungs, a blackish red spleen and a bone marrow of a darker red than normal. Pig. No. 27, dying twelve hours after .5 cubic centimeter of serum had been injected, showed in addition to the above a few hemorrhages in the intestine and hemorrhagic adrenals.

As controls, four pigs were injected with normal rabbit serum in quantities of from one to two cubic centimeters. Injection up to 1.5 cubic centimeters had no effect. Two cubic centimeters in one pig produced symptoms of severe dyspnea and general malaise followed by death at the end of four hours. Post-mortem showed a moderate hemopericardium and intense congestion of the lungs and spleen but no sign of spontaneous hemorrhage. The second pig receiving two cubic centimeters showed practically no discomfort. At the end of an hour there was a slight drop in the platelet count from 248,000 before injection to 192,000, but the bleeding time was unaltered. There was no further change.

Histological findings. — A histological study of the different organs showing hemorrhagic areas gave no information as to the manner in which these lesions were produced. Although

the hemorrhages were focal there was no definite evidence of infarction — such as occluded vessels. The indurated area present at the site of subcutaneous injection showed a fibrino-purulent exudate with extensive necrosis and proliferative changes occurring about the margin of the lesion.

The spleen in the guinea-pigs dying after injection of antiplatelet serum showed intense congestion, hemorrhages into the pulp and phagocytosis of the red cells by macrophages, some of which were of relatively huge size. In one animal dying after the injection of normal serum the congestion and hemorrhages were considerably less marked and there was no abnormal phagocytosis.

The findings in the bone marrow were inconstant. Marked engorgement of the blood channels was usually present. In some cases the megakaryocytes showed pyknotic and shrunken nuclei, but this change was also present in one guinea-pig which died after the injection of normal rabbit serum and it did not always occur in the animals which had received antiplatelet serum.

The effect of serum from animals injected with antiplatelet serum on platelets *in vivo* and *in vitro*. — Guinea-pig No. 21 received one cubic centimeter of antiplatelet serum intraperitoneally. Twenty-four hours later the animal had developed extensive purpura. The bleeding time was seventy-five minutes and the platelet count 24,000. The animal was bled and the serum used to inject other pigs. One cubic centimeter of this serum injected into the heart of one animal and into the peritoneal cavity of another produced no effect on the bleeding time or on the platelet count. The experiment was repeated with serum taken at the height of the reaction from a guinea-pig which had been inoculated into the heart. A slight reduction in platelet count and a very slight prolongation of bleeding time was observed one hour later in the pig receiving one cubic centimeter of this serum into the heart. The animal showed no other symptoms. All the animals remained well and none developed purpura.

Lytic tests with serum from animals injected with antiplatelet serum. — Sera from the guinea-pigs which were injected with antiplatelet serum were tested for agglutinating and lytic properties against normal guinea-pig platelets. The results were entirely negative. These sera produced lysis and agglutination only in dilutions stronger than one to six. In the same dilutions normal guinea-pig and rabbit sera cause lysis and agglutination. Antiplatelet serum produced the usual effects of lysis and agglutination on these platelets.

Local effect of antiplatelet serum. — It was thought that some light might be thrown on the mechanism by which the hemorrhages are produced in this condition by observing the local effect of antiplatelet serum on the small blood vessels of the mesentery. A guinea-pig was etherized, the abdomen opened and several loops of the intestine spread out on gauze moistened with warm salt solution. One cubic centimeter of antiplatelet serum was dropped slowly into one of these loops. The only local effect observed during the hour that the test was carried on was a moderate congestion of the area to which the serum was applied. However, at the end of forty-five minutes several small hemorrhages were seen in the two of the larger mesenteric glands and others could be produced at will by trauma. Soon petechiæ were observed on the abdominal wall and intestine, in areas quite distant from the point at which the antiplatelet serum had been applied. Some of these spots began to ooze. The bleeding time determined at this stage was twelve minutes. Death occurred shortly after this and autopsy revealed frequent infarcts in the lungs. The same result was obtained in another animal.

The animals present the complete picture of certain cases of purpura hemorrhagica in man. We found the diminished platelet counts and corresponding with this, the appearance of purpura, the prolonged bleeding time, the normal clotting time, and the failure of the clot to retract. Dr. George R. Minot will shortly report the complete study of a clinical case of purpura hemorrhagica. The serum of his case like

the sera of the guinea-pig injected with antiplatelet serum, did not destroy platelets in vivo or in vitro and failed to reproduce the condition.

SUMMARY AND CONCLUSIONS.

1. An antiserum for blood platelets of the guinea-pig had a strong agglutinating and lytic action on guinea-pig platelets. Furthermore the action was specific.
2. This reaction between antiplatelet serum and platelets would not occur without the presence of complement.
3. Injection of antiplatelet serum into guinea-pigs produced a condition typical in practically every way of the acute form of purpura hemorrhagica seen in man. There were numerous and profuse hemorrhages, a greatly delayed bleeding time, a marked diminution in the number of platelets, and a normal coagulation time but no retraction of the clot.
4. Histological examination of the hemorrhagic areas gave no information as to the manner in which the hemorrhages were produced.
5. Antiplatelet serum had no apparent local action.
6. The serum of guinea-pigs suffering from severe purpura had no effect on platelets either in vivo or in vitro.

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MORPHOLOGICAL CHANGES IN TISSUES WITH CHANGES IN ENVIRONMENT.

III. GASTRIC MUCOUS MEMBRANE IMPLANTED INTO THE INTESTINE.*

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A number of years ago Cade¹ called attention to changes in the structure of gastric mucous membrane of the dog and the cat following gastro-enterostomy. He noted that gastric glands at the site of the anastomosis between stomach and intestine were irregularly enlarged and often sinuous in appearance. Further, that the foveolæ of the gastric glands in this region appeared wider and deeper than normal, resembling in this respect the glands of the pyloric region of the normal stomach. The cells forming the glands were of one type and were like the chief cells found normally both in the neck of gastric glands and also in the glands lining the pyloric region of the stomach. Cells of this character were not stained with mucicarmine or muchematin, and did not show prozymogen or zymogen granules when stained with toluidene blue. From these observations Cade concluded that the cells of the gastric mucous membrane possessed the property of changing their morphology in response to new function and environment brought about by the artificial communication established between the stomach and intestine.

Subsequently Harvey,² also, studied the cellular changes in the mucous membrane of the stomach of dogs after gastro enterostomy. The animals were killed at intervals varying between two days and ten months. Harvey noticed changes in the gastric mucosa occurring regularly within a radius of seven millimeters of the anastomotic wound. Morphological changes were most extensive during the first

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three weeks following the operation; but during a period of six and a half months following operation the mucous membrane of the stomach was restored to its normal appearance. Harvey found that chief cells of the body of gastric glands which normally contain readily recognized granules of secretion were transformed into cells which possess the characters of a cell which forms mucus. He considers this change in the chief cells an actual transformation of the cells, probably temporary in character, as a restoration to a normal condition occurred in animals ten months after operation. In Harvey's experiment the principal change affected the chief cells of the mucosa; a transformation of parietal cells was not noted. Parietal cells at the site of anastomosis were frequently larger than normal and at times presented a vacuolated appearance.

In the series of experiments herewith reported, metaplastic changes resembling those described by Cade and Harvey at the site of gastro-enterostomy were noted in the gastric mucous membrane following the implantation into the small and the large intestine of flaps taken from the fundus of the stomach. The changes affected both the chief and the parietal cells. The transformation was found at times extensive in character, and present as late as fifteen months after the operation.

Method. — Dogs were used in all experiments. The stomach was exposed by an incision in the upper abdomen. A flap of the entire wall of the stomach was resected from the left end of the stomach, near the greater curvature, preserving at the same time the arterial and venous branches which extended from the spleen to the great curvature of the stomach. After the wound in the stomach was closed, the flap of the stomach, measuring usually from four to six centimeters, was then implanted into either the small or the large intestine, the anastomosis being made with silk sutures. In experiments where the flap of stomach was implanted into the lower part of the large intestine, a second incision of the

abdomen was made above the symphysis, and the flap of stomach properly protected with gauze was drawn from the region of the upper abdominal wound into the lower by means of a clamp introduced through the lower wound. In all, fifteen experiments were performed which were divided as follows: Implantation into the small intestine, twelve experiments: one animal was killed at the end of four days; four animals at the end of one month; two animals at the end of one and a half months; two animals after three and a half months; and one animal after six and a half, twelve, and fifteen months, respectively. Three experiments were done, two months, three months, and eight months in duration, in which the implantation was made into the large intestine.

In order to avoid repetition, only four of the experiments will be described in detail. The duration of these experiments was four days, three months, eight months, and fifteen months.

Experiment 1. — Dog, weight 9 kilos. An oval piece of stomach measuring 5 x 7 centimeters was resected from the region of the fundus and implanted into the intestine. Control tissue removed at the time of operation showed normal gastric mucous membrane.

Four days later the animal was killed and the grafted tissue was found in good condition. The mucosa of the implanted piece was slightly hyperemic; the epithelial surface appeared intact. Histologically, the epithelium of the implanted piece of stomach showed no changes excepting immediately in the region of the anastomosis with the intestine. Four or five glands nearest the line of anastomosis seem slightly dilated and lined with a low columnar cell, with an oval deep staining nucleus near the base of the cell. The cytoplasm of these cells does not contain the granules characteristic of the chief cells found in adjacent glands. Parietal cells are present but in small numbers; at times they appear somewhat distorted and slightly shrunken. The interglandular tissue shows capillaries considerably congested, but there is no increase in the connective tissue or in lymphoid cells.

Experiment 2. — Dog, weight 8.5 kilos. An oval flap of stomach measuring 5 x 7 centimeters was excised from the region of the stomach and implanted into the small intestine. Three months later in various parts of the grafted tissue striking histological changes in the morphology of the mucous membrane were noted; although in large part a normal mucous membrane was still present. In typical areas which exhibit

changes, glands and interglandular tissue do not show the delicate arrangement of structure seen in the normal mucous membrane (Figs 2 and 3). The glands appear wider, more irregular in form. The interglandular tissue appears thickened due to an increased accumulation of lymphoid cells and a dense appearance of the connective tissue. In areas affected with change in structure, both chief and parietal cells are absent in the glands, and in their place is found a columnar-shaped cell with oval nucleus at the base, presenting a clear, slightly granular cytoplasm. Mitotic figures are frequent both in cells at the surface and in those lining deep parts of the glands. The areas showing transformation of epithelium are scattered throughout the implanted tissue. In some sections the changes are confined to the zone near the line of suture with the intestine; sections taken from the grafted tissue at points distant from the anastomosis show, however, similar changes. Epithelium situated on the tops of folds appear particularly to be affected, due possibly to unusual exposure to traumatic conditions. Epithelium of glands situated deep in folds of the grafted tissue are as a rule well preserved, showing the normal delicately constructed glands containing chief and parietal cells.

Experiment 3. — Dog, weight 5 kilos. An elliptical piece of stomach measuring 6 x 3 centimeters was removed from the fundus of the stomach near the greater curvature and then implanted into the large intestine 8 centimeters from the anal end. Eight months later the grafted tissue was found in good condition; the pale folds of mucous membrane lying in a transverse direction, resembling in this regard the transverse arrangement of folds found in the adjacent large intestine. The microscopical study of the mucous membrane taken from various parts of the graft show a complete disappearance of chief and parietal cells from the glands. Instead, all glands are lined with cells resembling closely the epithelium at the surface of the mucous membrane. In appearance such cells are columnar and are free from secretory granules when stained with toluidene blue. Stained with mucicarmine, the outer third of the cells take a deep red color. This is particularly true of cells nearest the surface, while cells lining the deeper part of the gland react but slightly to this stain. Large goblet cells distended with mucus, resembling those in neighboring large intestine, are not found.

Lymphoid cells collected in follicles of considerable size are scattered plentifully throughout the mucosa and submucosa. These lymph follicles are apparently newly formed, inasmuch as they appear only occasionally in the control sections. The development of lymph follicles in the implanted tissue closely resembles the marked accumulation of lymphoid tissue in the mucous membrane of the gall bladder when this is implanted into the intestine, a condition described in earlier experiments.³ No noteworthy changes are seen in the connective tissue and elastic tissue of the graft in sections studied with Van Gieson and Mallory's stain. Blood vessels and the muscles of the graft are well preserved. Occasionally small groups of ganglion cells of normal appearance are found.

Experiment 4. — Dog, weight 6 kilos. An oval flap of stomach from the fundus measuring 5 x 6 centimeters was implanted into the middle of small intestine. Fifteen months later the animal was killed, and the mucous membrane presented in large part a normal appearance. Here and there, however, were found areas involving approximately from five to six glands where a mucous type of cells appeared in the glands in place of the usual chief and parietal cells. The changes are present mostly in areas near the line of union with the intestine and also at the tops of folds projecting into the lumen of the intestine.

Discussion. — Gastric mucous membrane removed from the fundus of the stomach and implanted into the small or the large intestine may undergo changes in structure characterized by a disappearance of chief and parietal cells normally found in the fundus. In place of chief and parietal cells a type of cell develops which resembles that normally found in the pyloric region of the stomach. These newly formed cells are of an indifferent character. They do not contain secretory granules but their distal end reacts strongly to stains for mucus. In addition to the changes in the character of epithelium the glands frequently show a greater irregularity in shape than normal. The foveolæ may show considerable deepening. The interglandular tissue of the implanted piece presents at times an increase in connective tissue and a great accumulation of lymphoid cells. In some experiments the structural changes in the implanted tissue affected only small areas of the graft and these appeared to be principally at the points of union between the grafted tissue and the intestine. Frequently the changes appeared at the tops of folds in the mucous membrane, which projected into the lumen of the intestine, where exposure to injury was greatest.

The cellular changes noted after implantation of mucous membrane of the fundus of the stomach into the intestine as here described bear a close resemblance to those observed by Cade and by Harvey, which occurred in the mucous membrane of the stomach of the dog and the cat at the site of gastro-enterostomy. Harvey describes the transformation of chief cells into mucous forming cells as occurring within a radius of seven millimeters of the anastomotic opening, the

transformation taking place during the first three weeks following the operation. The reverse change from indifferent cells of the mucous type without secretory granules to chief cells containing secretory granules was complete in his experiment which lasted six and a half months. In the experiments described in the present paper slight changes were noted as early as the fourth day, and well defined changes existed as late as fifteen months after operation. Not alone was a disappearance of chief cells noted, but of parietal cells as well. The transformation of grafted gastric epithelium was apparently complete in Experiment 3 eight months after the implantation was made into the lower part of the large intestine.

It is not a very unusual occurrence to find in the fundus of the human stomach areas where the epithelium has lost its normal character and has assumed the structure of intestinal epithelium. Cases in which this change has been present have been described by Hari,⁴ Schmidt,⁵ Boeckelman,⁶ and others. The occurrence in the human stomach of isolated areas resembling intestinal epithelium may be explained by assuming that the condition is either heterotopic caused by disturbances in embryonal development; or, that a metaplasia of fundic mucous membrane has occurred in the presence of a chronic inflammation. The experimental evidence, furnished by the changes in the gastric mucous membrane following gastro-enterostomy or the implantation of flaps of stomach mucosa into the small or the large intestine, suggest the possibility that in the human stomach a transformation of fundus mucous membrane into a form of epithelium resembling that of the intestine or the pylorus may readily occur as a result of abnormal conditions in the stomach, particularly those brought about by inflammatory processes.

The change exhibited by gastric mucous membrane from a highly complex to a simple form should be regarded as a metaplasia.

CONCLUSIONS.

Following implantation into the large or small intestine of flaps of gastric mucous membrane, a disappearance of chief and parietal cells may occur, the glands of the grafted tissue becoming lined with an indifferent type of cell resembling a mucus forming cell of the pylorus.

The transformation of gastric epithelium under conditions of implantation into the intestine is a metaplasia.

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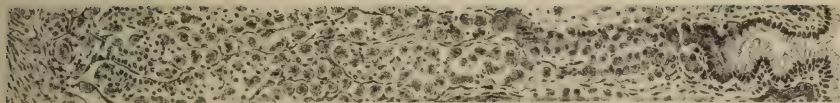
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DESCRIPTION OF PLATE XVII.

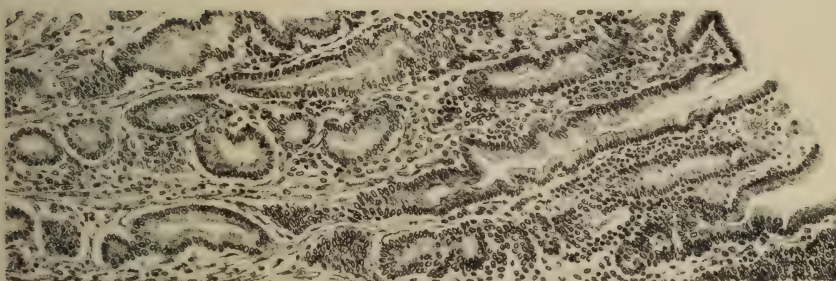
FIG. 1. — Normal mucous membrane from the fundus of the dog's stomach. x 300.

FIG. 2. — Mucous membrane of the fundus three months after implantation into the small intestine. The glands are irregular, the interglandular tissue is thickened, while the epithelium shows a mucous type of cell in the place of chief and parietal cells. x 300.

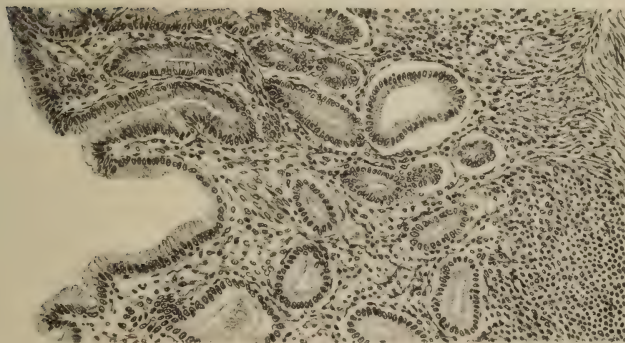
FIG. 3. — Mucous membrane of the fundus eight months after implantation into the lower part of the large intestine. No chief or parietal cells are seen, the glands being lined with an indifferent mucous type of cell. Lymphoid tissue is highly developed in the mucous membrane. x 300.



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LIPASE STUDIES:

III.

ON ACID PRODUCTION IN THE TISSUES WITH ESPECIAL REFERENCE TO URANIUM NEPHRITIS.*

CLARENCE QUINAN.

Introduction. — Although the formation of acid within the cellular structures seems to imply the existence of an intricate reacting system the essential elements of which may vary from time to time, nevertheless, an experimental examination of this mechanism by direct titration ought to be feasible. In other words, it should be possible at any stage of the acid-forming process approximately to determine the amount of free acid present. It is true that figures obtained in this manner could throw no light upon the finer changes which take place in autolysis, but at the same time they might serve a useful purpose in the study of gross pathology were they only to indicate a reaction trend in either direction.

Practically speaking, it is possible by means of a dilute standard solution of an alkali to make three fairly satisfactory measurements of free acid upon a definite mass of tissue, and in this way to gain an idea concerning the extent of intracellular ferment activity. In each instance the titration figure indicates total acidity. Fresh tissues seem invariably to contain pre-formed acid, and the amount of acid present in a unit weight of material is surprisingly constant in normal organs. This value constitutes the so-called initial acidity of fresh tissue. After the neutralized mixture has remained twenty-four hours in the incubator at 38° C. a second determination of acidity shows the increment due to continued autolysis, and this value also is fairly uniform. Finally, the acid set free by the action of the tissue ferment upon a simple ester may be measured. To a certain extent the value

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obtained in this manner seems to be characteristic of each tissue, provided that the experiments are conducted without loss and that the material is accurately weighed out.

The present paper is based entirely upon titration data. In the experiments which follow the object has been to compare such variations of total acidity as may occur in normal tissues with those to be noted in corresponding structures as the result of a fatal intoxication with uranium nitrate. A preliminary series of experiments had shown that the salts of uranium exert *in vitro* a depressing influence upon the intracellular ferment, and for this reason it was thought that a quantitative study of acid production in several tissues, simultaneously, especially in so far as the factor of lipase activity is concerned, might yield useful information and perhaps help to elucidate the pathology of nephritis.

Review of previous work on tissue acidity. — The general literature of the intracellular ferments is very rich, and to a great extent it is easy of access in the systematic treatises of Jacoby,⁶ Oppenheimer,¹⁰ and others. For an outline of the more important investigations on lipase the reader may consult an earlier paper of this series.¹⁴ A renewed discussion of that literature would be superfluous in this place, as the experiments which are about to be described do not differ in general scope from those in the paper referred to. However, as part of the data have to do with spontaneous acid production as it occurs in various tissues it will be necessary to review the literature which treats more particularly of autolysis.

It is probable that Fokker,³ in 1888, was the first to call attention to the fact that, in the absence of bacteria, acid develops in liver tissue. He interpreted the liberation of free acid as evidence of fermentative activity on the part of the protoplasm, and hence drew the conclusion that the production of acid constitutes "a common property of protoplasm — one which is possessed by all protoplasm."

Salkowski,¹⁹ however, two years later, made the first analytical study of the acids which result from autolysis. Flasks containing two hundred and fifty grams of fresh tissue in the

finely divided state, with twenty-five hundred cubic centimeters of chloroform water, were incubated at 38° C. for sixty hours, and the contents then were analyzed. Several acids were found in the mixtures. In discussing the formation of lactic acid in muscle he expressed the opinion that it is due to the activity of the living protoplasm. "It is not because it is dying that muscle tissue produces lactic acid," he declared, "but because it lives; and formation of the acid goes on only so long as the muscle lives." He continues: "Die Bildung von Milchsäure wäre demnach kein Absterbphänomen, sondern ein Lebensphänomen. Diese Anschauung hebt die Paradoxe auf, die darin liegt, dass ein und dieselbe Säure einerseits bei gesteigerter Leistung gebildet wird, andererseits beim Tode." Further to support the contention that lactic acid production is essentially a vital manifestation on the part of the muscle cells, he adduced the experimental observation that no lactic acid forms in a muscle "Brei," which has been treated with chloroform water.

Salkowski's results were confirmed by the observations of Jacoby⁵ in 1900, and again by those of Magnus-Levy⁹ in 1904. The latter author examined various tissues that had undergone aseptic autodigestion for periods varying from one day to six months. In the autolysates he found lactic, acetic, butyric, and other acids. Gases also were present. The occurrence of ready-formed lactic acid in "lebensfrischen Körperteilen" was emphasized. He thought it might be derived from carbohydrates, and stated that whilst paralactic acid usually is present in fresh tissues, the inactive form predominates after autodigestion. Ramond,^{16,17} also, inclined to the view that lactic acid may arise from a transformation of glycogen in the liver, but it was to the hydrolytic cleavage of the liver fats by the presence of lipase that he attributed the chief importance in the progressive formation of the acid.

The work of later investigators has cast a serious doubt upon the validity of Magnus-Levy's conclusions in at least two particulars. Wolbach and Saiki²⁴ found in the livers of twenty-two out of twenty-four dogs a peculiar anaërobic

bacterium which does not grow on ordinary media. They thought that this organism might play a part in lactic acid formation. And later, a perfectly sterile liver after forty-eight hours of autodigestion was analyzed by Saiki,¹⁸ and he found that of the total lactic acid present only a small proportion was of the inactive form. Jackson⁴ obtained similar results.

Pavy and Bywaters¹³ determined the total acidity from all sources in extracts of various organs. The fresh materials were rubbed up with sand, extracted with alcohol and strained through linen. Titrating the suspensions of tissue with deci-normal sodium hydroxide solution they found that cat's liver required forty-two and a half cubic centimeters, and cat's kidney thirty-seven and four-tenths cubic centimeters for each one hundred grams of material.

The foregoing papers deal with autolysis in the broader sense of the term; that is, the products of cleavage are derived from the tissue itself, and therefore the presence of acid implies a more or less extensive degradation of structure. All of them appear to emphasize spontaneous acid production as an outstanding characteristic of fresh tissue. Other workers, however, have attempted to trace out more definitely the origin of the lactic acid, and Yoshimoto,²⁵ Turkel,²³ Stein,²¹ Embden, Kalberlah and Engel,¹ Ssobolew,²⁰ Fletcher and Hopkins,² and Kondo,⁷ from various points of view, have made important contributions to the subject. A detailed review of these researches may be dispensed with in connection with the present report. In brief, however, the results of these investigators show that the production of lactic acid is due to enzymic activity and that, particularly in muscle press juice, the acid appears to be derived from an unknown "Vorstufe," the "Lactazidogen" of Embden. Isolated frog's muscle produces lactic acid, and in the presence of sufficient oxygen the intact muscle causes part of the newly-formed acid to disappear (Fletcher and Hopkins). Yoshimoto, Stein and others have observed that the ferment which produces the acid is powerfully depressed by chloroform.

Material and method. — Belgian hares were chosen for the purposes of this research, because it was necessary to select an experiment animal from which at least seven grams of fat-free kidney could be obtained. Forty-six were used in all. Of these, twenty-eight are included in the normal series, and fifteen in the series which received injections of uranium nitrate. The three remaining ones form a separate group. They appeared to be healthy animals, but in all three at autopsy unmistakable evidences of nephritis were found.

The liver, the kidneys, and some muscle tissue were removed from each animal and examined immediately. Here it will be necessary to describe a slight change in the method of preparing the tissues which was introduced because it seemed desirable to make separate tests of the kidney cortex and medulla. The procedure was as follows: The requisite amounts of cortex first were cut out and weighed, taking pains in so doing to avoid the underlying medulla. As a rule only the outer third of the cortex was used. The medulla then was exposed, all traces of cortical tissue were carefully trimmed away and the test quantity was weighed out. With normal kidneys this is a simple matter as the medulla is marked off from the cortex by a distinct line of contact and its surface may easily be laid bare. A very different picture presents itself, however, in the kidneys of animals which have received injections of uranium nitrate. In such kidneys the medulla is of a deep red or purplish red color, and the total mass of it is always greatly increased. When dissected free it is firm to the touch, and usually some fluid exudes from it. The cortical zone, on the other hand, is apt to be narrower than in the normal kidney, as though it were compressed by the outward-bulging medulla, and pin-point hemorrhages make their appearance upon a cross-section of it. With these kidneys extra care must be exercised in order completely to free the medulla from adherent cortical substance.

Excepting for the slight change in the technic just described, the four per cent suspensions of the different tissues were prepared and tested according to the writer's

method, of which details have been given elsewhere.¹⁴ In this research, for the sake of greater accuracy, the experiments were performed in triplicate, and three controls were carried through with each tissue

The last three Belgian hares of the normal series, and all of those which are included in the uranium nitrate series, were fed according to a definite plan of diet. This was done because experience has shown that some sort of dietary control is desirable in experiments with tissue ferments, and especially in those in which ethyl butyrate is employed as substrate. The experiment animals were treated as follows: Each Belgian hare was caged, supplied with water, and fed once daily twenty-five to thirty grams of fresh cabbage leaves. No other food was given. After four full days of this preparatory treatment, injections were begun, in case uranium nitrate was to be administered and, after the lapse of an additional fasting period of twelve hours, normal animals were killed.

The experimental observations which have made it seem necessary to adopt a standard diet when working with Belgian hare tissues will be discussed in the following section.

TABLE I. NORMAL LIVER.

For each test 1 gram of tissue, 25 cubic centimeters of distilled water, 5 cubic centimeter of toluol, and .5 cubic centimeter of ethyl butyrate.
Duration of experiments 24 hours. Temperature 38° C. The figures represent cubic centimeters of N/20 NaOH.

Belgian Hare No.	Weight.	Initial Acidity Per Gram of Liver. N/20 NaOH Required.	Ground Liver.				Controls.			Remarks.
			Cubic Centimeters of N/20 NaOH Required After 24 Hours.				Cubic Centimeters of N/20 NaOH Required After 24 Hours.			
			c.			Mean.	A. Autolytic. Not Heated.	B. Boiled. .5 cc. Eth. Buty.	C. Boiled. No Eth. Buty.	
			a.	b.	c.					
1	1600	1.4	40.2	38.8	37.9	38.9	2.3	1.3	0.0	Cabbage leaves and rolled oats. Moderate allowance.
2	1440	1.3	33.8	33.6	33.9	33.7	2.3	1.2	0.0	
3	1800	1.3	30.6	30.8	30.6	30.6	2.1	1.3	0.0	
4	1500	1.2	28.8	29.7	28.5	29.0	3.3	1.1	0.0	Cabbage leaves and rolled oats. Heavy feeding.
5	1920	1.3	28.2	27.5	27.8	27.8	1.8	1.2	0.0	
6	1800	1.4	35.7	35.1	35.5	35.4	2.4	1.1	0.0	
7	1500	1.1	26.4	26.1	26.2	26.2	1.0	0.9	0.0	Cabbage leaves and rolled oats. Heavy feeding.
8	1800	1.6	42.2	42.6	42.6	42.4	2.6	1.3	0.0	
9	2640	1.4	36.2	35.4	35.4	35.6	2.4	1.2	0.0	
10	2400	1.1	37.3	38.1	37.9	37.7	1.3	1.1	0.0	Cabbage leaves and rolled oats. Heavy feeding.
11	2000	1.3	33.8	33.1	32.9	33.2	1.6	1.1	0.0	
12	1200	1.2	31.0	31.6	31.2	31.2	1.4	1.5	0.0	
13	1680	1.5	38.8	39.3	39.1	39.0	2.3	1.3	0.0	
14	1080	1.6	43.6	43.4	43.3	43.4	1.8	1.3	0.0	

TABLE I. NORMAL LIVER. — *Continued.*

Belgian Hare No.	Weight.	Initial Acidity Per Gram of Liver. N/20 NaOH Required.	Ground Liver.				Controls, Cubic Centimeters of N/20 NaOH Required After 24 Hours.			Remarks.
			Cubic Centimeters of N/20 NaOH Required After 24 Hours.				A. Autolytic, Not Heated.	B. Boiled, .5 cc. Eth. Buty.	C. Boiled, No Eth. Buty.	
			a.	b.	c.	Mean.				
15	1350	1.6	35.5	35.3	35.6	35.4	1.8	1.3	0.0	Cabbage leaves and rolled oats. Heavy feeding.
16	1200	1.6	31.8	31.6	31.8	31.7	2.3	1.2	0.0	
17	1100	1.5	35.1	35.6	35.4	35.3	2.5	1.3	0.0	
18	1400	1.6	39.9	39.2	40.1	39.7	2.5	1.3	0.0	
19	1200	1.8	37.8	37.2	37.0	37.3	1.8	1.3	0.0	Cabbage leaves, carrots, and rolled oats. Heavy feeding.
20	1410	1.6	37.1	36.8	37.4	37.1	2.3	1.2	0.0	
21	1140	1.5	34.7	35.4	35.1	35.0	2.0	1.3	0.0	
22	1740	1.6	40.9	40.2	40.3	40.4	2.6	1.4	0.0	
23	1800	1.5	48.5	48.8	48.4	48.5	2.4	1.3	0.0	Cabbage leaves only. Moderate. Killed after 12 hours' fast.
24	1800	1.1	36.7	36.9	36.9	36.8	2.3	1.2	0.0	
25	1710	1.6	29.1	28.6	28.9	28.8	1.9	1.3	0.0	
26	1680	1.4	37.4	37.4	37.7	37.5	2.2	1.3	0.0	
27	1320	1.6	41.4	40.3	40.4	40.7	2.4	1.4	0.0	
28	1200	1.6	40.6	39.7	39.9	40.0	2.8	1.4	0.0	
Mean		1.4	36.0	2.1	1.2	0.0	

Experiments with normal tissues: (1) Liver. — As a general rule, even in Belgian hares of about the same weight, the livers vary in size, color, and firmness of texture. The differences in size especially may be very striking. It is probable of course that food is the determining factor, but the bulk of the liver may vary even in experiment animals which have been kept on a constant diet. Apparently some of them hydrolyze starch more rapidly than others. However that may be, the largest livers were found in Belgian hares which had been liberally supplied with carrots and rolled oats. In the normal series, Table I., Nos. 7, 10, 12, and 24 were livers of exceptional size. In physical characters the largest specimens are plump, the tissue is tender to cut and it is decidedly granular in consistency. As a rule they are rather pale in color, and the test suspensions were often milky or slightly opalescent in appearance. Medium sized and smaller livers seemed to be more nearly of the typical liver color, but after a brief period of starvation they may become quite dark in color and flabby in texture.

The concentration of pre-formed acid (initial acidity) is remarkably uniform in the substance of a liver, and weighed units give constant titration figures. But the results in Table I. show that in organs from different Belgian hares the amount of free acid in one gram varied from 1.1 to 1.6 in cubic centimeters of the standard alkali. One and four-tenths was the mean value obtained. It was observed that large livers yielded the lowest values.

In the normal series of twenty-eight Belgian hares, one gram of liver, in twenty-four hours, produced from its own substance an amount of autolytic acid equivalent, on the average, to two and one-tenth cubic centimeters of N/20 alkali.

The data in Table I. show that the ester-splitting activity of a liver is virtually constant for each gram unit, but it can be seen that this figure varies somewhat in different livers. Very large specimens as a rule give moderate or low values. Small, dark-colored, flabby livers always give the highest figures. The mean value 36.0, in Table I., probably is rather

low, as a majority of the experiment animals had received an almost unrestricted food allowance.

From the very beginning of the work the experimental results seemed to indicate that food in some way is able to affect the enzymic activity of liver tissue, but a large number of experiments had been made with livers from well-fed Belgian hares before some idea was gained as to the nature of its influence. The possibility that something in the food might be able to affect the titration figure indirectly was first suspected in the experiments with the livers Nos. 1 to 6, Table I., which plainly show a progressive diminution of the lipase values. The diet of the group of Belgian hares from which these livers were obtained had consisted of cabbage leaves and rolled oats, and the experiments covered a period of fourteen days. As all six of these animals were caged at the same time, and given the same food, the conditions were favorable to glycogen storage in the tissues. Under such a feeding regime, naturally, the last animal of the series would be likely to show the highest grade of glycogen enrichment. However, the cause of the progressive falling away in the lipase figures was not recognized at the time. And as the succeeding animals of the normal series were tested in groups of two to four, and the time period for each group did not exceed one week, the experimental conditions were not conducive to an explanation of the phenomenon. But, after some time, in the two groups 18 to 21, and 22 to 25, both inclusive, the same peculiarity was again noted, more or less definitely, and its relation to the diet seemed to be unmistakable.

Although no determinations of glycogen were made it was considered to be self-evident, from the appearance of the tissues, that the variations in size which were found to be characteristic of normal livers were largely due to the presence in the hepatic tissue of glycogen in greater or less abundance. Assuming this to be true, and taking into account the experimental observations already mentioned, namely, that perfectly normal livers of medium size yielded lipase figures, per gram of substance, that are not far from

37.0, and that, in general, the lipase figure seems to decrease in proportion as the liver increases in size, and vice versa, it was thought fair to assume, as a working hypothesis, that the ester-splitting activity of a liver cell would be most apt to remain constant if the diet were strictly controlled by weight. Glycogen-free liver probably would yield the highest and most consistent results were such material obtainable, but the practical difficulties in the way could hardly be overcome under ordinary experimental conditions. Having in mind the increase in the lipase figure which accompanies starvation, it was assumed further that, in the absolute sense, the ester-splitting capability of a cell may perhaps be one of its least variable properties. But this assumption seems to afford a possible explanation of the experimental findings, since, if it be supposed that the enzymic activity of a cell is fairly constant, it is obvious that in tests of liver substance by weight, the lipase value would become lower in proportion as the mass of the individual cell became greater. A progressive accumulation of glycogen in the cell, then, would cause a corresponding relative diminution of the lipase figure, per gram, because of the fact that the weighed test unit would contain fewer cells.

With the idea of putting this reasoning to the test it was planned to adopt an absolutely uniform diet for the experiment animals; preferably one containing a fixed minimum of glycogen-forming material. This aim was accomplished gradually. However, as the diet problem has an interesting phase, aside from that which concerns glycogen, a few experiments may be described at this point which deal with the lipolytic peculiarities of fresh cabbage leaves.

Experiments with cabbage leaves. — Tadokoru,²² in 1913, called attention to the fact that lipase is contained in the press juice of cabbages. He experimented with a series of plants and found that none but cabbage contained the ferment.

In the course of the present research many experiments have been made with cabbage leaves and also with carrots

and other vegetables, and it has been found that in the fresh state the two vegetables named exhibit well marked lipolytic activity.

FIG. 1. — *Hydrolysis of ethyl butyrate by the lipase of cabbage leaf. Comparison of mid-rib and parenchyma values in the normal condition, and after 24 hours' exposure to chloroform vapor. Figures represent cubic centimeters of N/20 NaOH.*

Description of Leaf.	Mid-rib.			Parenchyma.		
	Initial Acidity Per Gram.	Cleavage Acid Per Gram After 24 Hours.		Initial Acidity Per Gram.	Cleavage Acid Per Gram After 24 Hours.	
		A. Normal Leaf.	B. Exposed to Chloroform.		A. Normal Leaf.	B. Exposed to Chloroform.
Leaf 1. Large, dark green	0.4	0.8	0.6	1.2	2.9	1.9
Leaf 2. Large, light green	0.3	0.7	0.6	0.9	2.1	1.8
Leaf 3. Large, yellowish green . .	0.4	0.6	0.6	1.0	1.5	1.2

The results shown in Fig. 1 make it clear that the parenchyma of cabbage leaves is able to hydrolyze ethyl butyrate to a considerable extent. And it is interesting to note that in this respect the parenchyma figures are three times as large as those which were obtained with mid-rib preparations. A similar disparity in these structures is also to be seen in the figures for initial acidity. Thick, dark-green leaves seem to show the greatest, pale green or yellowish leaves the least, lipolytic activity.

It has been observed that various substances may inhibit the activity of the vegetable enzyme. Chloroform, for example, exerts a very depressing influence upon it, and cabbage leaves when they are exposed to the action of this drug, moreover, in less than two hours lose their natural green color and assume a uniform yellowish-brown tint. This effect may be produced either by plunging the cut end of the leaf into chloroform water, or by suspending the leaf in concentrated vapor of the anesthetic.

It is perfectly obvious, however, that the results obtained with the vegetable ferment are analogous to those which are yielded by animal lipase, since it has been pointed out in one of these studies¹⁵ that chloroform greatly reduces the ester-splitting activity of guinea-pig's liver.

FIG. 2. — *Hydrolysis of ethyl butyrate by the lipase of cabbage leaf. Comparison of the values obtained for parenchyma of normal leaves with those obtained for the same leaves after 4 hours' exposure to moist chlorine gas. The figures represent cubic centimeters of N/20 NaOH.*

Description of Leaf.	Initial Acidity Per Gram.	Cleavage Acid Per Gram After 24 Hours.	
		A. Normal Leaf.	B. Exposed to Chlorine.
Leaf 1. Large, thin, light green	1.3	2.2	1.1
" " " " " "	1.4	2.9	1.1
Leaf 2. Large, thin, dark green	1.6	3.4	1.0
" " " " " "	1.6	3.4	1.4
Leaf 3. Small, thick, dark green	1.7	3.6	1.7
" " " " " "	1.7	3.7	1.6
Leaf 4. Small, thick, very dark green .	2.0	4.5	1.8
" " " " " " " " .	2.0	4.6	1.7
Leaf 5. Large, light green	1.3	3.4	0.9
" " " " " "	1.2	2.9	0.9

The duplicate experiments which are included in Fig. 2 give a fair idea of the range of lipase values which are exhibited by different cabbage leaves, and they show that chlorine exerts a retarding influence upon the vegetable enzyme. The results of many experiments prove also that cabbage leaves offer very unequal resistance to the action of the pure gas, and that some of them are much more quickly decolorized by it than others. However, with reference to this element, the leaf ferment appears to behave like animal lipase, and the writer in another place¹⁴ has emphasized the fact that chlorine powerfully depresses the lipase of animal tissues.

The figures for lipolytic activity obtained with the ground substance of fresh carrots are very nearly the same as those noted in the experiments with cabbage leaves, but in the selection of a fresh vegetable with which to feed the experiment animals, the preference was given to cabbage leaves because of their relatively lower starch content. What part, if any, the ester-splitting ferment of the green cabbage leaf may play in the intestine or elsewhere in the body is unknown. A diet that is limited to a definite weight of this leaf, however, has certain advantages. At least, the possible sources of error due to variable amounts of glycogen-forming material and vegetable lipase in the food are minimized by it and rendered more nearly constant.

TABLE II. NORMAL KIDNEYS.

For each test 1 gram of tissue, 25 cubic centimeters of distilled water, .5 cubic centimeter of toluol and .5 cubic centimeter of ethyl butyrate. Duration of experiments 24 hours. Temperature 38° C. The figures represent cubic centimeters of N/20 NaOH.

Weight.	Initial Acidity Per Gram of Cortex. N/20 NaOH Required.	Initial Acidity Per Gram of Medulla. N/20 NaOH Required.	Cortex.				Medulla.		Controls.		
			Cubic Centimeters of N/20 NaOH Required After 24 Hours.				N/20 NaOH Required after 24 Hours.	Relative Lipolytic Activity.	A.	B.	C.
			a.	b.	c.	Mean.					
Belgian Hare No.	cc.	cc.					cc.	%	Autolytic. Not Heated.	Boiled. .5 cc. Eth. Buty.	Boiled. No Eth. Buty.
1 1600	1.4	29.9	28.1	28.8	28.9	2.1	1.3	0.0
2 1440	1.3	25.5	24.8	25.6	25.3	2.1	1.1	0.0
3 1800	1.3	26.8	25.9	26.4	26.3	1.4	2.0	0.0
4 1500	1.2	28.8	29.1	29.5	29.1	1.9	1.3	0.0
5 1920	1.3	27.8	28.6	27.6	28.0	2.3	1.1	0.0
6 1800	1.3	29.6	28.7	29.1	29.1	1.6	1.0	0.0
7 1500	1.7	29.9	29.2	29.8	29.6	2.3	1.4	0.0
8 1800	2.0	41.0	40.3	40.4	40.5	2.5	1.4	0.0
9 2640	1.2	24.5	23.6	24.2	24.1	2.5	1.2	0.0
10 2400	1.6	34.3	33.9	34.2	34.1	1.6	1.0	0.0
11 2000	1.6	29.9	29.3	29.7	29.6	1.6	1.0	0.0
12 1200	1.6	27.9	27.3	27.0	27.4	1.9	1.4	0.0
13 1680	1.5	0.9	29.4	30.6	30.4	30.3	19.3	63.7	2.2	1.3	0.0
14 1080	1.6	30.4	31.5	31.1	31.0	2.0	1.1	0.0

TABLE II. NORMAL KIDNEYS. — *Continued.*

Weight.	Belgian Hare No.	Initial Acidity Per Gram of Cortex. N/20 NaOH Required.		Initial Acidity Per Gram of Medulla. N/20 NaOH Required.		Cortex.				Medulla.		Controls. Cubic Centimeters of N/20 NaOH Required After 24 Hours.		
		cc.		cc.		Cubic Centimeters of N/20 NaOH Required After 24 Hours.				N/20 NaOH Required after 24 Hours.	Relative Lipolytic Activity.	A. Autolytic. Not Heated.	B. Boiled. .5 cc. Eth. Buty.	C. Boiled. No Eth. Buty.
						a.	b.	c.	Mean.					
15.	1350	1.5				25.3	25.4	25.3	25.3			1.6	1.1	0.0
16.	1200	1.4		0.8		25.3	24.1	24.9	24.7	15.4	62.3	1.8	1.2	0.0
17.	1100	1.6				26.6	26.3	26.8	26.5			2.1	1.3	0.0
18.	1400	1.6		1.2		26.6	26.3	26.1	26.3	15.6	60.0	2.0	1.3	0.0
19.	1200	1.8		1.2		26.4	26.7	26.8	26.6	17.2	64.6	2.2	1.3	0.0
20.	1410	1.7		1.1		28.6	28.0	28.1	28.2	16.4	58.1	2.0	1.3	0.0
21.	1140	1.6		1.2		27.0	26.7	27.2	26.9	16.4	60.9	2.3	1.4	0.0
22.	1740	1.5		1.1		25.1	25.8	25.3	25.4	16.6	65.3	2.6	1.1	0.0
23.	1800	1.4		0.9		37.3	38.5	37.8	37.8	26.4	69.8	2.1	1.2	0.0
24.	1800	1.6		0.9		37.8	38.4	38.2	38.1	24.5	64.3	2.3	1.7	0.0
25.	1710	1.9		1.1		28.0	27.8	27.9	27.9	15.8	56.6	2.2	1.6	0.0
26.	1680	1.4		1.0		26.7	26.8	26.7	26.7	16.3	61.0	2.3	1.2	0.0
27.	1320	1.4		0.8		27.2	27.4	27.6	27.4	17.8	64.9	1.8	0.9	0.0
28.	1200	1.4		0.8		28.6	28.8	28.2	28.5	17.8	62.4	2.2	1.3	0.0
Mean.....		1.5		1.0		28.8	18.1	62.6	2.0	1.2	0.0

(2) Normal kidney. — The normal cortex of Belgian hare's kidney is very tender to cut, and the cellular structure is easy to triturate in the mortar. The amount of pre-formed acid in one gram of it is very constant, and serial tests agree perfectly. This value varies a little in healthy Belgian hares, but the cortex figure usually is about 1.5. One and two-tenths was the lowest result obtained. It is possible that the experimental findings would show even smaller differences if the diet were strictly controlled. At any rate, in the present study the most even results were obtained with the tissues of animals that had received the standard diet. Apparently there are no exceptions to the rule that the medulla always contains less acid than the cortex. It cannot be said, however, that the acid concentration progressively diminishes in the direction from the free surface of the kidney toward its pelvis, because there seems to be no transitional zone where the values are intermediate. On the contrary, the change from the highly acid cortex to the less acid deeper lying structure is an abrupt one. Unfortunately, the present research was well under way before separate determinations were made of the amount of pre-formed acid in a weighed unit of medulla, but sufficient data are available (see Table II.) with which to establish the fact that the relation between the cortex and the medulla, in terms of free acid per unit of mass, is a remarkably constant one. For gram units of these tissues the ratio is 1.5:1.0. The difference here is one of degree only and not one of kind.

The mean value obtained for autolytic acid production, per gram, in twenty-four hours, was 2.0.

Both the cortex and the medulla of the kidney contain lipase. This fact was first pointed out by Loeper and Ficaï,⁸ who found, in tests of cat's kidney, that the lipolytic activity of the cortex varied from 19 to 25, whilst lower values, 9 to 16, were obtained for the medulla. They worked with monobutylin. The values obtained by the writer with ethyl butyrate are shown in Table II. It will be noted that the lipase figures for the cortex are fairly regular. The mean is 28.8, and the triplicate tests show good agreement. It is

probable that the gram lipase value in normal Belgian hare kidney cortex never falls below 24.0. The medulla invariably shows less lipolytic activity than the cortex. According to the figures in Table II., in the medulla the mean lipase value is 18.1. It is interesting to note, and it may be significant, that the cortex-medulla pre-formed acid ratio 1.5:1.0 is about the same as that which expresses the relative ester-splitting activity of these structures.

TABLE III. NORMAL MUSCLE.

For each test 1 gram of tissue, 25 cubic centimeters of distilled water, .5 cubic centimeter of toluol, and .5 cubic centimeter of ethyl butyrate. Duration of experiments 24 hours. Temperature 38° C. The figures represent cubic centimeters of N/20 NaOH.

Belgian Hare No.	Weight.	Initial Acidity Per Gram of Muscle, N/20 NaOH Required.	Ground Muscle.				Controls. Cubic Centimeters of N/20 NaOH Required After 24 Hours.		
			Cubic Centimeters of N/20 NaOH Required After 24 Hours.				A. Autolytic. Not Heated.	B. Boiled. 5 cc. Eth. Buty.	C. Boiled. No Eth. Buty.
			a.	b.	c.	Mean.			
1 . .	1600	1.6	5.8	5.5	5.4	5.5	0.6	1.1	0.0
2 . .	1440	1.9	5.7	5.2	4.8	5.2	0.6	1.1	0.0
3 . .	1800	2.5	5.4	5.5	5.8	5.5	1.0	1.1	0.0
4 . .	1500	2.2	5.9	5.9	6.0	5.9	0.8	0.9	0.0
5 . .	1920	2.7	5.5	5.3	5.8	5.5	1.4	0.9	0.0
6 . .	1800	2.3	6.8	6.9	6.6	6.7	0.9	1.0	0.0
7 . .	1500	2.3	6.6	6.1	6.3	6.3	1.2	1.0	0.0
8 . .	1800	2.4	5.9	5.9	6.0	5.9	0.5	1.0	0.0
9 . .	2640	2.6	7.8	7.9	7.3	7.6	0.6	1.0	0.0
10 . .	2400	2.3	7.1	6.6	7.2	6.9	1.0	0.9	0.0
11 . .	2000	2.5	6.4	6.6	7.1	6.7	1.1	0.8	0.0
12 . .	1200	2.2	5.1	4.8	5.2	5.0	1.4	0.9	0.0
13 . .	1680	2.7	5.8	5.4	5.5	5.5	1.0	0.9	0.0
14 . .	1080	2.2	6.9	7.3	6.7	6.9	0.7	1.0	0.0
15 . .	1350	2.3	5.9	5.7	5.7	5.7	1.0	1.1	0.0
16 . .	1200	2.1	5.1	5.0	4.9	5.0	1.0	0.9	0.0
17 . .	1100	2.2	6.2	5.9	5.8	5.9	0.9	0.9	0.0
18 . .	1400	2.1	6.3	5.9	5.9	6.0	0.8	1.0	0.0
19 . .	1200	2.8	6.3	6.7	6.4	6.4	0.8	1.1	0.0
20 . .	1410	2.3	6.1	6.2	6.2	6.1	1.1	0.9	0.0
21 . .	1140	2.5	7.2	6.9	7.0	7.0	1.0	1.0	0.0
22 . .	1740	2.1	5.7	5.5	5.6	0.9	0.9	0.0
23 . .	1800	2.3	7.3	6.9	7.3	7.1	1.2	0.9	0.0
24 . .	1800	1.9	6.1	5.6	5.6	5.7	1.2	1.1	0.0
25 . .	1710	2.3	5.4	5.8	5.3	5.5	1.2	0.9	0.0
26 . .	1680	2.1	5.6	5.4	5.5	5.5	0.7	0.9	0.0
27 . .	1320	2.0	5.8	6.6	6.3	6.2	0.4	0.9	0.0
28 . .	1200	1.9	6.2	6.0	6.1	6.1	0.7	1.0	0.0
Mean		2.2	6.0	0.9	0.9	0.0

(3) Normal muscle.—Experiments with fresh muscle tissue are chiefly remarkable for the extremely uniform figures which they yield for pre-formed acid. In the normal series, Table III., it will be seen that this value varies but little. As a rule each gram of muscle requires for complete neutralization of its acid about two cubic centimeters of the standard alkali.

The autolytic acid value is rather inconstant, and a mean value of 0.9 was obtained.

Fresh muscle accelerates the cleavage of ethyl butyrate only to a moderate extent, and the twenty-eight normal animals yielded a mean value of 6.0. However, probably because of variations in the glycogen content, well-nourished, plump muscles almost always give low values for lipase activity, whilst in states of emaciation the muscle values are high.

Experiments with uranium nitrate tissues: General remarks.—The Belgian hares of the uranium nitrate series were young, well-nourished animals of about fifteen hundred grams in weight. They were given injections of the salt, in one per cent solution, at the rate of three and a half milligrams per kilogram of body weight, and the course of the intoxication was studied in one pair at a time. The animals varied in their susceptibility to the action of the poison. Number 1 received four injections on successive days and was killed after eleven days. Number 2 received seven similar injections and died seventeen days later. The remaining thirteen animals each had three injections and lived on the average 4.2 days. Excepting Nos. 1 and 2 none lived over six days. A majority of the Belgian hares died from the effects of the drug, but Nos. 1, 6, 14, and 15 were killed. Usually, after the final injection, on the third day, a state of sluggishness and stupor supervened, with noticeable muscle relaxation, and, after lapsing by degrees into a condition suggestive of coma, the animals succumbed to the action of the poison within the next forty-eight hours. Some but not all of them became anuric after the last injection. On

section there was often a small quantity of fluid in the peritoneal cavity. Animals which survived the intoxication for six days or more continued to void a turbid urine. Usually they ate nothing after the fourth day. Always in such animals emaciation quickly became well marked, and on section the tissues looked dry. Although the Belgian hares varied in regard to the symptoms which followed the administration of the uranium salt, in all alike, at autopsy, characteristic changes were found in the kidneys, the deeper lying structures of which were always congested and dark red in color. Aside from the conspicuous alterations in the medulla, often the other gross findings were unimportant. Chemical examination of the various organs, however, gave results which seem to be characteristic, and which are very different from those obtained with normal structures. The essential points of difference will be made clear by a brief description of the experiments with these tissues.

TABLE IV.

Uranium nitrate intoxication in 15 Belgian hares. Values obtained for liver tissue. Experiment conditions constant — see Table I.

Belgian Hare No.	Initial Acidity Per Gram of Liver, N/20 NaOH Required.	Ground Liver.				Controls. Cubic Centimeters of N/20 NaOH Required After 24 Hours.		
		Cubic Centimeters of N/20 NaOH Required After 24 Hours.				A. Autolytic. Not Heated.	B. Boiled. .5 cc. Eth. Buty.	C. Boiled. No Eth. Buty.
		cc.	a.	b.	c.			
1 . .	1.6	37.0	36.7	37.0	36.9	2.5	1.3	0.0
2 . .	1.6	37.5	37.9	38.0	37.8	2.5	1.4	0.0
3 . .	0.9	28.8	28.6	28.6	28.6	1.2	1.1	0.0
4 . .	1.3	28.8	28.3	28.2	28.4	1.4	1.2	0.0
5 . .	1.0	28.2	27.4	27.1	27.5	1.3	1.0	0.0
6 . .	1.1	33.4	33.7	34.5	33.8	2.1	1.2	0.0
7 . .	1.1	29.2	29.2	28.9	29.1	1.3	1.1	0.0
8 . .	1.1	26.6	26.7	25.7	26.3	1.2	1.0	0.0
9 . .	1.2	34.0	33.6	. . .	33.8	0.9	1.1	0.0
10 . .	1.0	28.1	28.4	28.6	28.3	1.4	1.0	0.0
11 . .	1.2	28.8	28.9	29.4	29.0	1.1	1.3	0.0
12 . .	1.0	25.8	25.7	25.2	25.5	1.2	1.0	0.0
13 . .	1.0	28.9	29.2	28.4	28.8	1.3	1.1	0.0
14 . .	1.3	33.6	34.0	33.5	33.7	1.6	1.1	0.0
15 . .	1.2	34.1	33.6	33.3	33.6	1.6	1.3	0.0
Mean.	1.1	30.7	1.5	1.1	0.0

(1) Uranium nitrate liver. — In all experiments with uranium nitrate livers and kidneys it was observed that the test suspensions were much more translucent in appearance than is the case with normal tissues. The end reactions also were sharper. Apparently, as a result of the toxic nephritis, the fluids of the body became hypertonic, and hence the suspended proteins in the tests, because of the presence of electrolytes in increased amounts, more nearly approached the condition of solution. As it has already been stated, the poisonous effects of uranium nitrate were manifested unequally in different Belgian hares. Two exceptional cases may be mentioned in which the duration of the

intoxication was more than twice the average. Of these, in No. 1, see Table IV., profuse hemorrhage from the lower bowel was an unusual symptom. At autopsy the liver proved to be small, it was dark in color, and when it was triturated it gave off a peculiar, disagreeable odor. Belgian Hare No. 2, on the other hand, remained free from hemorrhages, in spite of the fact that it received almost twice as much of the poison as No. 1 and, though the liver was small and flabby, otherwise it was not peculiar in any way. Suppression of the urine did not occur in either of these animals. From the figures in Table IV. it will be observed that the results of the liver tests in Nos. 1 and 2 are about the same as those which characterize normal livers; that is, the figures for acid production are normal. With the exception of Nos. 1 and 2, however, in every other member of the series the acid-producing faculty of the liver cells was markedly depressed in uranium nitrate poisoning, and it is obvious from the data in Table IV. that the amount of pre-formed acid in a unit of liver tissue, after a fatal intoxication with this salt, is far below the normal figure. A mean value of 1.1 was obtained in the series of fifteen livers, and the individual differences are small. The mean value found in normal livers was 1.4.

The figures in Table IV. show, also, that the mean autolytic acid value, after uranium nitrate poisoning, fell away from 2.1, which is the normal mean, to 1.5.

Turning now to the results of the tests with ethyl butyrate, it is evident that here, as well, the values are considerably below those obtained with normal tissues, and the data in Table IV. show that the general average for the fifteen Belgian hares was only 30.7. The normal mean value was 36.0.

There seems to be no doubt, therefore, in view of the harmonious results obtained under different experimental conditions, that the acid-producing faculty of the liver cells is profoundly depressed in uranium nitrate intoxication.

TABLE V.
*Uranium nitrate intoxication in 15 Belgian hares. Comparison of the lipolytic values obtained for kidney cortex and medulla.
 Experiment conditions constant — see Table II.*

Belgian Hare No.	Initial Acidity Per Gram of Cortex, N/20 NaOH Required.		Initial Acidity Per Gram of Medulla, N/20 NaOH Required.		Cortex.				Medulla.		Controls.		
	cc.	cc.	cc.	cc.	Cubic Centimeters of N/20 NaOH Required After 24 Hours.				N/20 NaOH Required after 24 Hours.	Relative Lipolytic Activity.	Cubic Centimeters of N/20 NaOH Required After 24 Hours.		
					a.	b.	c.	Mean.			A. Autolytic, Not Heated.	B. Boiled, 5 cc. Eth. Buty.	C. Boiled, No Eth. Buty.
1	1.1	0.9	23.5	22.5	23.3	23.1			14.0	60.6	1.4	1.3	0.0
2	0.6	0.4	29.6	29.6	29.8	29.6			15.1	51.0	1.7	1.1	0.0
3	1.0	0.8	17.1	17.0	16.8	16.9			13.9	82.2	1.8	1.1	0.0
4	1.1	0.8	21.8	21.0	22.4	21.7			13.2	60.8	2.1	1.2	0.0
5	0.7	0.6	19.6	20.2	21.2	20.3			15.9	78.3	1.2	0.9	0.0
6	0.7	0.5	23.0	23.8	23.5	23.4			14.8	67.5	1.4	1.0	0.0
7	1.0	0.9	17.3	17.6	16.8	17.2			13.2	76.6	1.4	0.9	0.0
8	0.8	0.6	18.9	17.9	17.7	18.1			14.4	80.0	0.9	1.0	0.0
9	0.9	0.7	27.6	26.7	27.0	27.1			19.8	73.0	1.7	1.0	0.0
10	0.8	0.6	19.5	19.4	19.2	19.3			14.6	75.1	0.8	0.9	0.0
11	0.8	0.5	20.3	20.4	20.8	20.5			15.8	77.0	0.9	1.1	0.0
12	0.8	0.6	17.6	17.4	18.1	17.7			13.9	78.5	1.2	1.0	0.0
13	1.0	0.7	21.9	22.0	21.9			16.4	74.8	1.5	1.4	0.0
14	0.9	0.7	19.6	19.8	19.6	19.6			13.8	70.4	1.4	0.9	0.0
15	1.0	0.8	16.8	16.3	17.0	16.7			13.6	81.4	1.5	1.1	0.0
Mean . . .	0.8	0.6	20.8			14.8	72.4	1.3	1.0	0.0

(2) Uranium nitrate kidney. — As might have been anticipated, the toxic influence of uranium nitrate is particularly evident in the results of the experiments with kidney tissues. And it is at once to be seen from the figures in Table V. that the acute nephritis which follows the administration of this salt is characterized by an overwhelming depression of acid production both in the cortex and the medulla. The results of the direct titrations of free acid in weighed units of kidney substance, perfectly fresh material being used in each instance, show not a single exception to this rule. Fresh nephritic tissues are invariably less acid than the corresponding normal ones. By comparing the cortex-medulla pre-formed acid values in Tables II. and V. it will be seen that the figures for free acid in uranium nitrate kidneys show reductions in excess of forty per cent, the mean values having fallen from 1.5–1.0 in the normal to 0.8–0.6 in the morbid tissues. It would be natural to infer, from the fact that a red and swollen kidney medulla is the salient autopsy finding in an animal that has succumbed to an intoxication with uranium nitrate, that a higher degree of ferment depression would prevail in the tubular structures than in the cortex. Such, however, is not the case, for almost always it has been found that the cortex suffers more than the medulla in this respect. As a matter of fact, in spite of the greatly retarded acid production in both tissues, the normal ratio is not much altered.

So far as the formation of autolytic acid is concerned, in uranium nephritis, the inhibition of ferment action was well marked, and from the mean normal value of 2.0, which represents the amount of acid produced by a gram of tissue in twenty-four hours, this value fell to 1.3.

The marked depression of spontaneous acid production both in the cortex and the medulla, which is revealed by the figures in Table V., finds an exact counterpart in the results which were obtained with an artificial substrate. The mean values found with ethyl butyrate were 20.8 and 14.8, for the cortex and medulla, respectively. The corresponding normal values were 28.8 and 18.1. Here, again, it is obvious that

ferment inhibition is slightly more pronounced in the cortex. The figures for cortical lipase activity in Table V., taken as a whole, are fairly uniform, and inasmuch as a majority of the experiment animals died from the effects of the uranium salt, it seems to be clear that so far as the kidney tissues are concerned the critical depression point for the ester-splitting ferment is from twenty-five to thirty per cent below the normal figure. However, some experiments with different organs presently to be described, which concern the peculiarities of acid production as they occur in connection with spontaneous nephritis, will show that an inhibition of the kidney enzyme alone, to this extent, does not, of necessity, lead at once to a fatal issue.

TABLE VI.

Uranium nitrate intoxication in 15 Belgian hares. Values obtained for muscle tissue. Experiment conditions constant — see Table III.

Belgian Hare No.	Initial Acidity Per Gram of Muscle, N/20 NaOH Required.	Ground Muscle.				Controls. Cubic Centimeters of N/20 NaOH Required After 24 Hours.		
		Cubic Centimeters of N/20 NaOH Required After 24 Hours.				A.	B.	C.
		a.	b.	c.	Mean.	Autolytic. Not Heated.	Boiled. .5 cc. Eth. Buty.	Boiled, No Eth. Buty.
1 . .	1.4	6.4	6.2	6.2	6.2	0.6	1.0	0.0
2 . .	0.9	7.5	6.8	6.6	6.9	0.7	1.0	0.0
3 . .	1.2	5.7	5.3	5.3	5.4	0.4	1.0	0.0
4 . .	1.5	5.0	5.0	5.1	5.0	0.3	0.9	0.0
5 . .	0.9	4.7	4.8	4.7	4.7	0.4	0.9	0.0
6 . .	0.7	5.2	5.5	5.4	5.3	0.4	0.9	0.0
7 . .	1.1	5.1	5.0	5.0	5.0	0.2	1.0	0.0
8 . .	1.1	4.9	4.9	5.0	4.9	0.3	1.0	0.0
9 . .	1.0	5.7	5.7	5.7	5.7	0.5	1.0	0.0
10 .	0.9	7.0	6.6	6.6	6.7	0.4	0.9	0.0
11 . .	1.0	7.0	7.5	7.2	7.2	0.4	1.0	0.0
12 . .	0.7	4.9	4.8	5.0	4.9	0.4	1.0	0.0
13 . .	1.1	4.9	5.0	5.2	5.0	0.4	0.9	0.0
14 . .	2.1	5.9	5.8	5.6	5.7	0.8	1.0	0.0
15 . .	1.2	5.9	6.0	6.0	5.9	0.4	1.0	0.0
Mean.	1.1	5.6	0.4	0.9	0.0

(3) Uranium nitrate muscle. — In all but one of the Belgian hares which were injected with uranium nitrate, see Table VI., the values for pre-formed acid, per gram of muscle, show a mean reduction of exactly fifty per cent. From 2.2, which is the normal mean, this value fell to 1.1. This tremendous inhibition of acid production in the skeletal muscles is virtually a constant finding in uranium nitrate intoxication. The tissue is perfectly normal in appearance, but when the figure for total acidity is determined by a direct titration the acid content of the muscle tissue is found to be only about half the usual normal amount.

A loss even greater than this manifests itself in the values for autolytic acidity. In normal muscles the mean value per gram is 0.9, whereas in uranium intoxication 0.4 was the value noted.

In striking contrast to the examples of diminished acid formation in muscle tissue just described, the experiments with ethyl butyrate show that the ester-splitting activity of muscle is not appreciably influenced by the poison. The mean value here is about the same as that which was recorded in the normal series, Table III.

Acid production in spontaneous nephritis. — In the course of the present research well-marked examples of spontaneous nephritis were discovered in three Belgian hares. Outwardly these animals seemed to be in good condition. They were active and showed no signs of disease. In all three of them, however, large, nephritic kidneys were found. Those of No. 30, see Fig. 3, an animal that weighed eighteen hundred grams, were typical. The two kidneys weighed together 28.050 grams. On the surface they were mottled with yellowish-white patches. The medullary tissue on section was greatly increased in bulk, and it was rather tough to cut. Over the congested, purplish-red, central part of the organ the cortex lay as a thin covering, yellowish in color.

FIG. 3. — *Spontaneous nephritis in Belgian hares. Experiment conditions constant. Figures represent cubic centimeters of N/20 NaOH.*

Belgian Hare No.	Liver.			Kidney.					Muscle.		
	Initial Acidity Per Gram.	Mean Net Cleavage Acid Per Gram After 24 Hours.	Autolytic Acid in 24 Hours. Control "A."	Initial Acidity Per Gram of Cortex.	Initial Acidity Per Gram of Medulla.	Mean Net Cleavage Acid Per Gram After 24 Hours.		Autolytic Acid in 24 Hours. Control "A."	Initial Acidity Per Gram.	Mean Net Cleavage Acid Per Gram After 24 Hours.	Autolytic Acid in 24 Hours. Control "A."
						Cortex.	Medulla.				
29 . .	1.6	36.3	2.1	1.1	19.9	2.3	2.2	5.4	0.8
30 . .	1.4	29.3	1.4	1.1	0.9	21.1	14.7	1.3	2.7	5.7	1.0
31 . .	1.6	38.4	2.4	0.9	0.4	23.3	14.8	1.3	1.1	6.1	0.8
Mean.	1.5	34.6	1.9	1.0	0.6	21.4	14.7	1.6	2.0	5.7	0.8

The principal results recorded in the experiments with various tissues from these nephritic animals are brought together in Fig. 3. It will at once be noted that in so far as the kidneys are concerned the figures are very nearly the same as those which were obtained in uranium nitrate kidneys. It is evident, therefore, that in spontaneous just as in experimental, nephritis, acid production in the kidney tissues is diminished, and in both cases the figures for pre-formed acid and those for ester cleavage show about the same amount of inhibition. A comparison of the liver and muscle values, however, makes it clear that the two forms of nephritis are only alike in what concerns acid production in the kidneys themselves. They are not alike, it appears, with respect to the influence which the disordered kidneys are able to exert upon the other tissues. Thus it will be observed that the liver and muscle pre-formed acid values were not far from normal in the three cases of spontaneous nephritis, Fig. 3, whilst these values in uranium nephritis animals were very greatly diminished. While it is true that acid production in the kidneys suffers about equally both in the naturally occurring and the experimental nephritides, attention should be called to the fact that in the former an extensive overgrowth of connective tissue is to be noted in all parts of the kidney, and the actual increase in the mass

of the medulla may be enormous. In toxic nephritis, on the other hand, if large amounts of uranium nitrate have been administered, the animal dies before proliferation of the interstitial tissue can take place.

PROTOCOLS OF THE URANIUM NITRATE BELGIAN HARES.

B. H. 1. — Weight 1,500 grams. Caged March 13, 1915, on cabbage diet. 0.0035 milligram of uranium nitrate, pro kilo, March 17, 18, 19, and 23. Hemorrhage from bowel March 27. Killed March 28. Tissues dry. No fluid in belly. Liver dark brown; small; peculiar, disagreeable odor.

B. H. 2. — Weight 1,450 grams. Caged March 16, 1915, on cabbage diet. 0.0035 milligram of uranium nitrate, pro kilo, March 20, 22, 23, 29, April 1, 2, and 3. Found dead and still warm April 6. Autopsy findings as in No. 1. Emaciation.

B. H. 3. — Weight 1,600 grams. Caged April 9, 1915, on cabbage diet. 0.0035 milligram of uranium nitrate, pro kilo, April 13, 14, and 15. Found dead April 16. A little clear fluid in belly. Voided no urine last two days.

B. H. 4. — Weight 1,480 grams. Caged April 9, 1915, on cabbage diet. 0.0035 milligram of uranium nitrate, pro kilo, April 13, 14, and 15. Found dead April 16. A little clear fluid in belly. Voided no urine last two days.

B. H. 5. — Weight 1,500 grams. Caged April 21, 1915, on cabbage diet. 0.0035 milligram uranium nitrate, pro kilo, April 25, 26, and 27. Found dead April 30. Little fluid in belly. Liver much above the average in size.

B. H. 6. — Weight 1,450 grams. Caged April 21, 1915, on cabbage diet. 0.0035 milligram of uranium nitrate, pro kilo, April 25, 26, and 27. Killed May 1. Moribund at the time. A little fluid in belly. Voided no urine last two days.

B. H. 7 — Weight 1,500 grams. Caged May 3, 1915, on cabbage diet. 0.0035 milligram of uranium nitrate, pro kilo, May 7, 8, and 9. Found dead May 11. A little clear fluid in belly. No urine voided after the third day. Large liver.

B. H. 8. — Weight 1,480 grams. Caged May 7, 1915, on cabbage diet. 0.0035 milligram of uranium nitrate, pro kilo, May 13, 14, and 15. Found dead May 16. No fluid in belly.

B. H. 9. — Weight 1,550 grams. Caged May 7, 1915, on cabbage diet. 0.0035 milligram of uranium nitrate, pro kilo, May 15, 16, and 17. Found dead May 19. No fluid in belly. Small, flabby liver, dark in color.

B. H. 10 — Weight 1,500 grams. Caged May 18, 1915, on cabbage diet. 0.0035 milligram of uranium nitrate, pro kilo, May 23, 24, and 25. Found dead May 25. Emaciation. No fluid in belly. Clear urine in bladder contains a trace of albumen but no sugar.

B. H. 11. — Weight 1,450 grams. Caged May 18, 1915, on cabbage diet. 0.0035 milligram of uranium nitrate, pro kilo, May 23, 24, and 25. Found dead May 26. Emaciation. No fluid in belly.

B. H. 12. — Weight 1,480 grams. Caged June 3, 1915, on cabbage diet. 0.0035 milligram of uranium nitrate, pro kilo, June 8, 9, and 10. Found dead June 12. A little clear fluid in belly. Large liver.

B. H. 13. — Weight 1,300 grams. Caged June 3, 1915, on cabbage diet. 0.0038 milligram of uranium nitrate, pro kilo, June 8, 9, and 10. Died June 12. Very little clear fluid in belly.

B. H. 14. — Weight 1,800 grams. Caged June 7, 1915, on cabbage diet. 0.0035 milligram of uranium nitrate, pro kilo, June 12, 13, and 14. Killed June 18. Not much evidence of intoxication. Voided quantity of turbid urine on last day. No fluid in belly.

B. H. 15. — Weight 1,500 grams. Caged June 7, 1915, on cabbage diet. 0.0035 milligram of uranium nitrate, pro kilo, June 12, 13, and 14. Killed June 18. Profuse hemorrhages from bowel June 17 and June 18. No fluid in belly.

Discussion. — It has been shown in the present research that fresh, normal kidney, liver, and muscle tissues contain acid and that with the exception of the kidney the acid concentration is uniform in each organ. The cortex and the medulla of the kidney in this respect behave as different tissues, and it is possible to express the relation which they sustain to one another by the ratio 15 : 1.0.

There seems to be no doubt that acid production is dependent upon ferment activity, because if a suspension of ground tissue is heated to 100° C. for half an hour, after an exact neutralization of its free acid, and then it is incubated for twenty-four hours at 38° C., no more acid forms; but if the preliminary heating is omitted a further liberation of acid takes place. Moreover, both the cortex and the medulla of the kidney, when in the form of a four per cent suspension, are able to accelerate the hydrolysis of ethyl butyrate and in the cleavage of this ester the titration figures exhibit the same ratio which characterizes the pre-formed acid values. But the experimental data show that similar enzymic activities are manifested by liver and muscle tissues. Hence it seems to follow that the various structures of the body contain a common ferment, probably lipolytic in character and reversible in its action, which exerts an influence upon acid

production within the cells, and which is able to accelerate the cleavage of a simple ester.

The experimental results clearly prove that uranium intoxication is characterized by diminished formation of acid, not only in the tissues of the kidney, where the poison induces a visible alteration, but also in those of the liver and the skeletal muscles. In all of these tissues the acid-forming mechanism gives evidence that it is under a powerful inhibitive influence. It may be questioned, however, whether the reduced acid values which have been noted after the administration of uranium nitrate are to be attributed to a retardation of the ferment by this salt alone, although it is true that *in vitro* it depresses the intracellular ferment. The extent of the depression and its uniform character seem to make this explanation of the phenomenon an unlikely one. If now the violent inflammation of the kidney medulla which follows an injection of the salt is taken into account, together with the fact that the tubular structures are believed to be concerned in the elimination of various salts, and if it is recalled that many electrolytes are able to depress the lipolytic ferment, the experimental findings it is thought support the view that in uranium intoxication, owing to some change in the tubular epithelium which is induced by the poison, sodium chloride and other substances are retained in the body and depress the intracellular ferment. As a matter of fact, the figures indicate that after a fatal intoxication with the uranium salt, inhibition was about as pronounced in the liver as in the kidneys. Pathologists as a rule have confined their attention to the alterations which this poison induces in the kidneys, but Opie¹¹ and Oertel¹² have described cellular changes in the liver as well and, according to Oertel, in uranium poisoning the liver changes bear a more or less close resemblance to the changes in the kidneys.

It is perhaps possible that the degree of ferment inhibition may stand in some relation to the severity of the intoxication. At any rate, to judge from the figures which were obtained in the study of spontaneous nephritis, it is doubtful whether acid production in the tissues is marked by an

extreme retardation unless the poison is present in sufficient quantity to render the tubular epithelium impermeable.

While there is no evidence to show that the enzymic "Tatigkeit" of the tissue cells, which reveals itself by spontaneous acid formation, is specific so far as the function of an organ is concerned, at the same time the low acid values which were noted in the experiments with nephritic tissues suggest the idea that the proper performance of function perhaps is conditional upon ferment activity, and the data of this research seem to show that in healthy organs this value, in terms of pre-formed acid per gram of substance, remains nearly constant.

SUMMARY.

1. Fresh, normal tissues are acid in reaction when tested with phenolphthalein and in weighed units the pre-formed acid values are constant.

2. It is suggested that a lipolytic ferment in some way plays a part in the process of spontaneous acid production as it occurs in the cellular structures.

3. The mean values obtained for pre-formed acid, per gram of tissue, in twenty-eight normal Belgian hares were: Liver 1.4, kidney cortex 1.5, kidney medulla 1.0, and muscle 2.0.

4. Liver, kidney, and muscle tissues yield constant and characteristic values when tested with ethyl butyrate.

5. In uranium nephritis acid production is profoundly depressed, not only in the kidneys but also in the liver and the skeletal muscles. These tissues give low values for pre-formed acid, autolytic acid, and ester-splitting activity.

6. The pre-formed acid values per gram of tissue, after uranium nitrate intoxication, were: Liver 1.1, kidney cortex 0.8, kidney medulla 0.6, and muscle 1.1.

7. It is suggested that the reduced acidity of the tissues, which is characteristic of experimental nephritis, is due to an inhibition of intracellular ferment activity by sodium chloride and other substances which are retained in the body in consequence of some change in the tubular epithelium induced by uranium nitrate.

8. It is pointed out that in chronic spontaneous nephritis the pre-formed acid values of the kidney cortex and medulla are below normal.

9. It is suggested that the functional integrity of an organ implies a certain degree of ferment activity which, in terms of pre-formed acid per gram of tissue, can be roughly measured.

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THE COMPLEMENT FIXATION REACTIONS OF THE BORDET-GENGOU BACILLUS.*

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Serological studies of the relationship of the Bordet-Gengou bacillus to hemoglobinophilic bacilli have been made by various investigators since Bordet and Gengou, in 1906, claimed the discovery of the etiological cause of whooping cough in the bacillus bearing their name.

In their first article on the subject¹ Bordet and Gengou stated that an influenza antigen used in tests on the serum of pertussis convalescents (which with an antigen of the Bordet-Gengou bacillus invariably inhibited hemolysis) did not inhibit hemolysis, from which they concluded that the influenza bacillus has no etiological relationship to whooping cough and that the complement fixation test could be utilized to differentiate these two bacterial species. They found the antigen of the influenza bacillus to be anticomplementary but not the antigen of the Bordet-Gengou bacillus. Arnheim^{2,3} also tested the serum of pertussis cases with both Bordet-Gengou and influenza antigens, and with the latter all results were negative. In working with immune rabbit serums he found that deviation in the presence of homologous antigens always took place even in small amounts of antibody and that Bordet-Gengou and influenza strains did not cross-fix. Wollstein,⁴ working with immune rabbit serums, found the serums of rabbits immunized with various strains of the Bordet-Gengou bacillus to fix complement in the presence of Bordet-Gengou antigen but not influenza antigen, while influenza immune serum bound complement with an influenza antigen and not with the Bordet-Gengou antigen. Baecher and Menschikoff⁵ successfully used the complement fixation reaction to confirm the identity of freshly isolated strains of the Bordet-Gengou bacillus.

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Odaira,⁶ working with immune rabbit serum, found complement fixation a valuable means of differentiating the Bordet-Gengou bacillus from the influenza bacillus. Shiga, Imai and Eguchi⁷ state that the Bordet-Gengou bacillus can be clearly differentiated from influenza bacilli by means of complement fixation. In testing the serum of rabbits immunized with the Bordet-Gengou bacillus, they never obtained complete inhibition with an influenza antigen; but in testing influenza serum they did in some instances, not in others, obtain complete inhibition with a Bordet-Gengou antigen, though not in so high a dilution of serum as with an influenza antigen. Inaba⁸ described antibody content titrations of the serum of five rabbits immunized with five strains of the Bordet-Gengou bacillus. The serums were tested against antigens of ten strains of the Bordet-Gengou bacillus and practically the same amount of inhibition occurred in all tests. Winholt⁹ has recently reported on the absence of cross-fixation between the Bordet-Gengou bacillus and the influenza bacillus.

The objects of our investigation were to confirm the above results concerning the differentiation, through complement fixation tests, of the Bordet-Gengou bacillus from hemoglobinophilic bacilli, to differentiate the organism from certain forms, culturally and morphologically atypical, obtained from the sputum of pertussis cases and to determine the inter-relationship of the various strains of the Bordet-Gengou bacillus. Work on the serums of pertussis cases has been described in another article.¹⁰

Strains. — Fourteen strains of the Bordet-Gengou bacillus have been used in this work, four strains of the Bordet-Gengou bacillus morphologically and culturally atypical, and nine strains of hemoglobinophilic bacilli. The Bordet-Gengou strains P.D. and M and the hemoglobinophilic strain BI₂ were obtained from other laboratories. The hemoglobinophilic strains 747, 11, and z were isolated from the spinal fluid of cerebro-spinal meningitis cases. All the other strains were obtained from the sputum of pertussis cases. Most of

the strains were isolated and identified, morphologically and culturally, by Dr. A. W. Williams, who, in regard to strains isolated from whooping cough cases, has stated:¹¹ "The majority of the strains isolated by us have been shown to be a distinct species from the hemoglobinophilic bacilli and from certain other bacilli morphologically similar to the Bordet-Gengou bacillus. Not only are cultural characteristics different, but these strains produce specific antibodies in experimental animals which give positive results with the complement fixation test."

Technic. — The volume of all our complement fixation tests is one-tenth that of the classical Wassermann, .5 cubic centimeter instead of five cubic centimeters. For a hemolytic system we have used sheep cells in a five per cent suspension, rabbit amboceptor and guinea-pig complement in a ten per cent dilution. The balance of the system has been daily obtained by means of an amboceptor titration with .1 cubic centimeter of ten per cent complement incubated one hour in a water bath at 37° C.; and an amount of amboceptor between one and two units thus determined has been used to sensitize cells for filling antigen and antibody content titrations, the exact amount of amboceptor depending on the rapidity of hemolysis, the amount hemolyzing in about thirty minutes being chosen.

The relationship of strains has been determined by means of antigen and serum titrations. (The technic of titrations is that described in "Pathogenic Micro-organisms," by Park and Williams, 1914, pp. 184-187) All titrations have been incubated in a water bath at 37° C. one-half hour before the addition of sensitized cells, one hour, afterwards. The readings have been made after the settling of the cells, usually the following day, the tests meantime standing in the ice-box. If an immediate reading was desired, the tubes were centrifugalized. All serum has been freshly inactivated before use by heating one-half hour at 56° C. Each antigen has been standardized by titrating with a previously standardized homologous immune serum, so diluted that .01 cubic

centimeter contained about two antibody units. If no serum of the same strain was available, serum of some closely related strain was used. When neither antigen nor serum was standardized, the antigen was first titrated with .01 cubic centimeter of undiluted serum, and with the antigen unit thus obtained the serum was titrated. After obtaining the serum unit the antigen was re-titrated with a suitable amount of serum. Each antigen has been tested in a sufficient number of dilutions (1 in 10, 1 in 100) to determine the smallest amount that with a minimum amount of immune serum completely inhibits hemolysis; this amount we designate as the Antigen Unit. Immune serum has been titrated with antigen so diluted that .1 cubic centimeter contained about two antigen units, according to standardization with homologous serum. Each serum has been tested in a sufficient number of dilutions (1 in 10, 1 in 100) to determine the smallest amount that with a minimum amount of antigen completely inhibits hemolysis; this amount we designate as the Antibody Unit.

Antigens. — Antigens have been prepared according to various methods. The media used were 1–500 coagulated horse blood veal agar (blood added to agar at 90° C.) for influenza and older pertussis strains, Bordet-Gengou for recently isolated pertussis strains and salt free veal agar, neutral to phenolphthalein, for the atypical Bordet-Gengou strains. In the beginning of the work a modification of Schwartz and McNeils' method of preparing gonococcus antigen was employed, — a forty-eight-hour growth (on neutral, salt free veal agar) in pint blake bottles was washed off with neutral distilled water, heated in a water bath at 56° C. for two hours and filtered through a Berkefeld. This method of autolysis proved to be satisfactory for atypical Bordet-Gengou strains and fairly so for the Bordet-Gengou, especially if the growth was scraped off with a platinum spud and deposited in distilled water, thus preventing the extraction from the medium of substances that render an antigen non-specific. As, however, the pertussis organisms

are not easily autolyzed, the extracts thus obtained were weak. Shaking for two to eighteen hours was found to add greatly to the efficiency of pertussis antigens. Of the various combinations of shaking and heating that have been tried the following has given the best results and is now used in the preparation of all pertussis antigens: A forty-eight-hour growth on Bordet-Gengou medium is scraped off and deposited in neutral distilled water and put in an electric shaker for three to four hours. The temperature is then brought up to 56° C. in a water bath and the emulsion is left in a thermostat at 56° C. overnight. The following day it is centrifugalized and the supernatant fluid or Berkefeld filtrate is used as antigen, after being made isotonic by the addition of nine per cent saline.

In determining the comparative value of different antigens, the range of fixation with homologous immune serum — *i.e.*, the difference between the anticomplementary dose and the antigen unit — and specificity are the chief points to be considered. As far as specificity is concerned, the different methods of antigen preparation that have been tried have given practically the same results. There is such great individual variation in antigens of the same strain made by the same method that a comparison of methods is difficult. If the value of an antigen is expressed numerically a comparison is facilitated. The scheme suggested by Walker and Swift¹² is convenient. If the anticomplementary dose is divided by the antigen unit, a figure is obtained that may be called the Index of fixation. The larger the index the stronger the antigen, hence the method of preparation is best for the production of a strong antigen that has the highest median index, *e.g.*, One antigen of strain P.D. made March 25, 1914, autolyzed by heat only, had an anticomplementary dose of .2 cubic centimeter and an antigen unit of .05 cubic centimeter, so the fixation index was $\frac{.2}{.05} = 4$. The same method used before, on October 29, 1913, on the same strain, resulted in an antigen with a unit of .015 cubic centimeter and not at all anticomplementary in .4 cubic centimeter, the largest amount tested. The index of this antigen

was therefore $\frac{.4+}{.015} = 26.6+$. Another P.D. antigen made on April 4, 1914, autolyzed by shaking two hours and heating at 56° C. one hour, had an anticomplementary dose of over .4 cubic centimeter (exact amount not determined) and a unit of .005, *i.e.*, an index of $\frac{.4+}{.005} = 80+$. If the fixation indices of all antigens made by each method are determined, the median index may be taken as a basis for comparison. The Bordet-Gengou antigens extracted by heat only have a median index of $8+$, those extracted by shaking and heat have a median index of $26+$; thus for the preparation of Bordet-Gengou antigens a combination of shaking and heating seems preferable to heating alone. For the preparation of influenza antigens neither method is really satisfactory. A number of antigens have had so short a range as to be useless. The shaken antigens have been more anticomplementary than the unshaken and have had a median index of 2 only. Such an antigen is too poor to be reliable and has not been used in cross-fixation tests. Most of the influenza antigens autolyzed by heat only have not been anticomplementary in .4 cubic centimeter and the median index is $4+$, which indicates a fairly good antigen. To obtain reliable results in any complement fixation work an antigen should be used that has an index at least of 3. Results are likely to be more specific if the fixation index is high. As to the antigens made from the atypical strains, not enough have been shaken to demonstrate the value of that method, but extraction by heat has produced antigens with a median index of 30, which is perfectly satisfactory. It is evident that each organism to be used in complement fixation work requires special study as to the preparation of antigen. The methods used in this work we do not consider ideal and modifications are constantly being tried. The antigens made, however, have been sufficiently satisfactory for at least a preliminary study of the complement fixation reactions of the Bordet-Gengou and allied organisms. By the use of more refined methods it might be possible to obtain antigens so specific that strains apparently identical would be differentiated.

Immune serum. — Immune serum was obtained by inoculating rabbits with *B. pertussis*, typical and atypical strains, and *B. influenza*. The injections were chiefly with living forty-eight-hour cultures intraperitoneally, a few with vaccines and antigens subcutaneously and intravenously respectively. Trial bleedings were made before inoculations and about once a week after the third inoculation, until the serum showed a high enough titer for complement fixation tests. The dosage began with the 1/4 to 1/8 slant of recently isolated cultures and 1/2 slant of older cultures. Some of the atypical strains were very virulent and the first dose was from 1/100 to 1/50 slant. The dose was increased according to the weight of the animal. As a rule the increase was gradual until the rabbit received the surface growth from four to six one hundred cubic centimeter bottles. The intraperitoneal injections were once a week, the subcutaneous and intravenous twice a week. The rabbits were bled to death on the seventh or eighth day after the last inoculation.

Of one hundred and four rabbits tested while normal, six only have shown complete inhibition in .02 cubic centimeter, the maximum amount of serum tested. One of these had a unit of .005 cubic centimeter, increasing to .003 cubic centimeter after three inoculations of *B. pertussis*, one a unit of .006 cubic centimeter, increasing to .003 cubic centimeter after three inoculations of *B. pertussis*, one a unit of .008 cubic centimeter, increasing to .005 cubic centimeter after three inoculations of *B. pertussis*, and the other three a unit of .02 cubic centimeter. Of the last group the antibody content increased to .002 cubic centimeter in one instance after three inoculations of *B. pertussis*; the other two rabbits (inoculated with *B. influenza*) showed no increase in complement fixing substances after three inoculations.

Eighty-one rabbits have been immunized by the live culture method, with an average of eight inoculations. One of these never showed complete inhibition in even .02 cubic centimeter of serum. One had an antibody unit of .0002 cubic centimeter after nine inoculations. If each serum is

considered at the time when the antibody content was highest the median unit is found to be .002 cubic centimeter.

To determine the rate of increase in complement fixing substances following inoculations of live cultures of pertussis, atypical pertussis and influenza bacilli, the median antibody unit has been determined for each inoculation. After one and two inoculations no trial bleedings were made. After three inoculations fifty-two rabbits were bled and the serum was titrated against homologous antigens or those most closely related. When a serum has been tested against several homologous antigens, slightly different results have sometimes been obtained, for no apparent reason; and to exclude the error arising from a choice of records, all such tests have been included in determining the median. Thus the number of titrations cited in Table I. exceeds the number of serums titrated. Experience has shown that serum with an antibody unit of .003 cubic centimeter is sufficiently strong to give specific results in cross-fixation tests and to retain its complement fixing substances for a considerable length of time, hence such a serum is considered good.

TABLE I
COMPLEMENT-FIXING VALUE OF IMMUNE RABBIT SERUM
AFTER VARIOUS INOCULATIONS WITH LIVE CULTURES OF BORDET-GENGOU,
ATYPICAL BORDET-GENGOU AND INFLUENZA BACILLI.

Number of Inoculations	Titrations	Median Unit Greater than	Number with unit of 0.005 cc. or less w. Good	Percentage Good
3	70	0.02 c.c.	14	20.
4	96	0.004 "	45	46.8
5	108	0.004 "	45	41.6
6	116	0.004 "	53	45
7	92	0.004 "	41	44.5
8	137	0.004 "	59	43.
9	68	0.003 "	45	66.1
10	58	0.002 "	37	63.7
11	40	0.002 "	31	77.5
12	16	0.0025 "	11	68.7
13	15	0.002 "	8	53.3
14	9	0.0009 "	8	88.8
15	2	0.00055 "	2	100.

If all the good serums are added, according to the number of inoculations, the percentage of good serums resulting from each inoculation is obtained (Table I.). According to both the median unit and the percentage of good serums there is a great increase in the development of complement fixing substances after the fourth inoculation. From the fourth to the ninth inoculation little change occurs. With about the ninth inoculation there is another increase, which gradually continues. The number of serums tested beyond the thirteenth inoculation is too small to warrant conclusions. It appears, therefore, that four inoculations are likely to result in the production of a good serum.

Eleven rabbits were immunized with vaccine, ten inoculations, on an average. The weakest serum showed no complete inhibition in .02 cubic centimeter, the strongest showed complete inhibition in .0006 cubic centimeter, the median unit was .007 cubic centimeter. Three rabbits were immunized with the bacterial extract antigens, with an average of six inoculations. The weakest serum had an antibody unit of .005 cubic centimeter, the strongest an antibody unit of .002 cubic centimeter, the median unit was .004 cubic centimeter. From so small a number no conclusions can of course be drawn. There is no doubt but that the live culture method employed is superior to the vaccine method for the production of complement fixing substances.

The immune serums have been preserved by freezing and within a period of one year little change in the antibody content has been observed.

Cross titrations. — The cross titration work has extended over a considerable period. In working with so many strains it has not been feasible to have on hand at one time satisfactory antigens and satisfactory serums of all strains. Thus no one serum has been tested against antigens of all strains, nor has any one antigen been tested against serums of all strains.

TABLE II
RESULTS OF ANTIGEN TITRATIONS

RESULTS OF ANTIGEN REACTIONS																								
Antigen Strains		Serum Strains																						
		Bordet Gengou										Atypical					Hemoglobinophilic							
Bordet Gengou	PD	M	55	81	93	95	98	100	110	114	121	154	C	I	31	10	33	35	37	87	747	B1 ₂	Z	
	PD	+	+	+	+	+	+	+		+	+		-	±	±	±	-	-	-	±	-	-	-	
	M	+	+										-	±	±	-	-	-	-	±	-	-	-	
	55	+	+	+	+	+	+	±	+	+			-	-	±	-	-	-		±	-	-	-	
	81	+	+	+	+	+	+	+	+	+					-	-	-	-	-		-	-	-	
	93	+	+	+	+	+	+	+	+	+					+	+	+	-	-		±	-	-	
	95	+	+	+	+	+	+	+	+	+	+	+	±	±	±	-	-	-		±	-	-	-	
	98	+	+	+		+	+	+	+	+			-	±	±	-	-	-	-	±	-	-	-	
	100	+	+												±	±	+	-	-		-	-	-	
	110	+	+		+		+																	
	114		+			+		+		+											+			
	121	+	+	+	+	+	+	+		+	+	+					-				-	+	-	
	141	+	+	+	+	+	+	+	+	+	+	+									+	+		
	154	+	+	+	+	+	+	+	+	+	+	+	-	±	±	-		-	-	±	-	-	-	
	155	+	+	+	+	+	+	+	+	+	+	+								±	-	-	-	
Atypical	C	-	-	-	-	-	-	-	-	-			+	-	-	-	-	-	-	-	-	-	-	
Bordet Gengou	I	±	-	-	-	-	-	±		±			-	±	±	-	-	-	-	-	-	-	-	
	31	±	±	±	±	-						±	-	±	±	±	-	-	-	-	-	-	-	
	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-	-	-	-	-	-	-	
Hemoglobinophilic	33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	±	±	±	+	
	35	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	±	±	±	±	+	
	37	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	±	±	±	±	-	
	87	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	±	±	-	-	-	
	188	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-				-	+	+	+	
	747	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				-	+	+	+	
	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	±	±	±	±	±	
	B1 ₂	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	±	±	±	±	±	±	±	
	Z	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	±	±	±	±	±	±	±	

TABLE III
RESULTS OF SERUM TITRATIONS.

	Antigen Strains																											
	Bordet-Gengou														Atypical BC					Hemoglobinophilic								
Serum Strains	PD	M	55	81	93	95	98	100	110	114	121	141	154	155	C	I	31	10	33	55	37	87	188	747	11	B ₁₂	Z	
Bordet-Gengou	+	+	+	+	+	+	+	+	+		+	+	+	+	+	-	±	±	-	±	-	-	-	-	-	-	-	-
M	+	+	+	+	+	+	+	+	+		+	+	+	+	+	-	-	-	-	±	-	-	-	-	-	-	-	-
55	+	+	+			+	+		+	+	+	+	+	+	+	-	-	+	-	±	-	-	-	-	-	-	-	-
81	+		+	+	+	+	+		+	+	+	+	+	+	+	-	-	-	-	±		-	-	-	-	-	-	-
93	+		+	+	+	+	+		+		+	+	+	±	+	-	-	-	-		-	-	-	-	-	-	-	-
95	+	+	+	+	+	+	+		+		+	+	+	+	+	-	-	+	-	±	-	-	-	-	-	-	-	-
98	+	+	+	+	+	+	+		+		+	+	+	+	+	-	-	+	-	±	-	-	-	-	-	-	-	-
100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	±	-	-	-	-	-	-	-	-
110			+		+	+	+		+		+		+	+		-	-	-	-	±	-	-	-	-	-	-	-	-
114	+	+	+	+	+	+	+		+		+		+	+		-	-	-	-		-	-	-	-	-	-	-	-
121	+		+	+	+	+	+		+	+	+	+	+	+	+	-	±	-	-	±	-	-	-	-	-	-	-	-
154	+			+	+	+	+		+		+	+	+	+	+	-	±	-	-		-	-	-	-	-	-	-	-
Atypical	C	-			-	-	-				-	-			-	+	-	-	+	-	-	-	-	-	-	-	-	-
Bordet-Gengou	I	±		±	±	±	±		±		±	±	±	±		-	±	±	±	±	-	-	-	-	-	-	-	-
31	+	-	-	+	+	+	+		+		+		+	+		-	-	±	±	±	-	-	-	-	-	-	-	-
10	-	+	-	-	-	-	-	±	±		-	-			-	-	-	±	±	-	-	-	-	-	-	-	-	-
Hemoglobinophilic	33	-	-	-											-	-	-	-	-	-	+	+	±	±	±	-	±	-
	35	-	-	-											-	-	-	-	-	-	+	+	±	±	±	-	±	-
	37	-	-	-	±	±	±				±	±			-	-	-	-	-	-	-	+	+	±	±	-	-	-
	87	-	-	-											-	-	-	-	-	-	±	±	±	±	±	-	±	-
	747	+	+	+	+	±	±	±	±	±	±	±	±	±	±	-	±	±	±	±	±	±	±	±	±	±	±	±
B ₁₂	-	-	-	-					-		-				-	-	-	-	-	±	±	±	±	±	±	±	±	±
Z	+	+	±	+	+	+	+			+	±	±	±	±		-	±	±	±	±	-	-	-	-	-	-	-	-

Tables II. and III. are composites, made up from the total number of titrations. In almost every instance the results recorded have been confirmed by repeated tests with serums of the same strain from different rabbits and with different antigens of the same strains. + denotes complete inhibition of hemolysis; — denotes incomplete or no inhibition of hemolysis; ± denotes that irregular results have been obtained, either complete inhibition on some occasions and incomplete on others, all tests being apparently equally valid, or that complete inhibition has occurred in a lower dilution than with the homologous strain. The significance appears to be the same in either case, *i.e.*, a relationship of strains not identical. For example, serum P.D. fixed in practically the same amount in a 1 in 100 dilution with four different Bordet-Gengou strains (with 93, 121, and 155 in .0003 cubic centimeter; with 98 in .0004 cubic centimeter); with four different influenza strains and with three atypical Bordet-Gengou strains it gave no complete inhibition in the maximum amount of serum tested, .02 cubic centimeter; with the atypical I it fixed in .003 cubic centimeter, *i.e.*, in a 1 in 10 dilution. Between .0003 cubic centimeter or .0004 cubic centimeter and .003 cubic centimeter there is so great a difference that these two strains, P.D. and I, cannot be considered homologous, though the relationship is rather close. Titrations of Serum I lead to the same conclusion. Antigen titrations of P.D. and I show less difference between the strains but enough to confirm the results of the serum titrations. Other Bordet-Gengou strains have shown a similar relationship to the atypical I and to 31, which stands in about the same relationship to I as to the Bordet-Gengou strains. A similar relationship exists between the Bordet-Gengou strains and the two influenza Strains 747 and z. Serums of Strains 747 and z have fixed with antigens of Bordet-Gengou strains, but no antigen of Strain 747 or z has fixed with a Bordet-Gengou serum, which confirms the experiments of Shiga, Imai and Eguchi.⁷ The other influenza strains studied and the atypical C are entirely distinct, according to complement fixation, from the

Bordet-Gengou strains studied. The atypical 10 has cross-fixed slightly with Bordet-Gengou strains.

No consistent difference in the Bordet-Gengou strains themselves has been demonstrated, but the subject is still under investigation. Certain influenza strains that appeared to be identical (33-35, BI_2 -11) were found to be capable of separation if a larger amount of complement was used in the test. For example, No. 107 (strain BI_2) when titrated with one unit of complement and one unit of amboceptor fixed in .0003 cubic centimeter with its homologous antigen, in practically the same amount (.0002 cubic centimeter) with Strain 11, in .002 cubic centimeter with Strains 33 and 35, and not even in .02 cubic centimeter with the hemoglobi-nophilic Strain 37 or the Bordet-Gengou Strain 55. On increasing amboceptor to two units, the results were practically the same. With the use of one and one-half units of complement and only one unit of amboceptor there occurred a corresponding weakening of both strain and group fixation; the antibody unit with the homologous Strain BI_2 was .0005 cubic centimeter; with Strain 11, .0004 cubic centimeter; with Strains 33 and 35 the smallest amount that gave complete fixation was .005 cubic centimeter. By increasing the amboceptor to two units a further change in both strain and group fixation was brought about, the unit with BI_2 falling to .0009 cubic centimeter, that with 33 to .004 cubic centimeter, and that with 35 to .008 cubic centimeter. With two of complement and one of amboceptor, fixation occurred with BI_2 and 11 only, in .0008 cubic centimeter and .001 cubic centimeter respectively. With two units of complement and two of amboceptor, the units were .0009 cubic centimeter with BI_2 and .002 cubic centimeter with 11, which may be identical with BI_2 . No tests have been made with more than two units of complement and amboceptor. Similar tests have been made with Bordet-Gengou strains but, even with two units of complement and two of amboceptor, a consistent difference in the strains has not appeared. With a greater increase in one or the other reagent a differentiation may be possible.

Some of the inconsistent results obtained, as in cross titrations of Bordet-Gengou and closely related atypical strains, have undoubtedly been due to variations in the hemolytic system, as an accurate standardization of neither amboceptor nor complement is obtained by titrating amboceptor with ten per cent complement, as we have done. This crude method of obtaining the balance of the hemolytic system seems, however, to give as uniform results as the use of a definite number of complement and amboceptor units, due probably to variations in the fixability of complement.

Another reason for the lack of uniformity in results arises from the fact that equivalent amounts of antigen or serum were not always used. About two units of antigen, according to a recent standardization with two units of homologous immune serum, were used in serum titrations and about two units of serum in antigen titrations; but as antigen gradually deteriorates and as the complement varies in fixability the antigen unit may change from one day to the next. Unfortunately, it is not always practicable to titrate all antigens before beginning a day's work to assure the use of equivalent amounts of antigen in all serum titrations. By combining the results of a large number of titrations and excluding all that appear questionable we feel that valid conclusions may be drawn from the work.

CONCLUSIONS.

1. The separation by morphological and cultural characteristics of the typical Bordet-Gengou bacillus from atypical strains of the Bordet-Gengou bacillus and from the influenza bacillus has been confirmed by complement fixation tests.
2. *B. pertussis* is somewhat related in its complement fixation characteristics to at least two strains of the influenza bacillus and to at least two atypical pertussis strains, but is differentiable.
3. Immune serums of Bordet-Gengou strains have been found to cross-fix with antigens of Bordet-Gengou strains only. Immune serums of some influenza strains have cross-fixed with Bordet-Gengou as well as with influenza strains.

4. Twelve strains of *B. pertussis* isolated in this laboratory from cases of pertussis have given complement fixation reactions similar to each other and similar to the two strains obtained from other laboratories and may be considered practically homologous.

5. By the use of more highly specific antigens or larger amounts of complement and hemolytic amboceptor than those ordinarily employed a differentiation of individual strains of *B. pertussis* may be possible.

6. To obtain an immune serum of high complement-fixing-antibody content, four intraperitoneal inoculations of a living culture of *B. pertussis* or *B. influenzæ* may be sufficient, nine or more inoculations are advisable, as a decided rise in the antibody content curve occurs after nine inoculations.

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FURTHER EXPERIMENTAL STUDIES ON THE INHERITANCE OF
SUSCEPTIBILITY TO A TRANSPLANTABLE TUMOR, CAR-
CINOMA (J. w. A.) OF THE JAPANESE WALTZING
MOUSE.*

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In a previous investigation one of us has shown that a carcinoma of the Japanese waltzing mouse grows on implantation in a high proportion of this race and in no instance in the common mouse. The first filial generation of hybrids produced by cross-breeding these two varieties were almost as susceptible as the Japanese waltzing mice, but their offspring, both in the second and third filial generation were all non-susceptible. These findings could not at that time be accounted for on the basis of Mendel's law, especially if susceptibility was considered as a unit factor, or on the basis of any other hypothesis of inheritance. The growth of the tumor in a single F_2 mouse subsequent to the publication of these results suggested that an occasional susceptible animal might occur in later generations, providing sufficient numbers were obtained.

The object of the present investigation was to determine, if possible, the mode of inheritance by which such results as these are obtained, and for this purpose larger numbers of hybrids derived from other stocks of common mice have been tested. Before considering subsequent results it will be useful to review briefly the work already accomplished in this field of research.

Cuénot, whose series of papers on color inheritance in mice constitute a classic in genetics, has in collaboration with Mercier attempted to ascertain whether or not any one color variety of mouse is markedly more favorable than others to the growth of implants of a given tumor. In that all the color varieties with which they worked were found to

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be essentially equal in their reaction towards the tumor, their results may be briefly described as negative.

Leaving the question of color and turning to that of race, marked differences in susceptibility are found. Haaland, Loeb, Loeb and Fleischer, Tyzzer and Higuchi have shown that race is an important factor in the transplantation of tumors. The larger part of the results obtained by these investigators have shown varying percentages of positive and negative animals after inoculation, according to the race used. Thus, Tyzzer reports a series of inoculations in which four races of mice were used. These races were (1) tame mice from a Providence dealer, (2) tame mice from Buffalo-Cambridge stock, (3) inbred laboratory stock, and (4) Japanese waltzing mice. The Jensen tumor used in inoculation grew in 11.7 per cent of the Providence mice, in 41.6 per cent of the Buffalo-Cambridge stock, in 80 per cent of laboratory stock, and in none of the Japanese waltzing mice. With the Ehrlich tumor the same four races show 26.3, 64.3, 91.7, and 37.5 per cent respectively of positive animals.

The susceptibility of F_1 hybrids obtained by crossing the laboratory stock with Japanese waltzing mice was also tested. In these tests the results were as follows:

JENSEN TUMOR.

Laboratory stock mice, tumors in.....	80%
F_1 hybrids, tumors in	10%
Japanese waltzing mice, tumors in	0%

EHRlich TUMOR.

Laboratory stock mice, tumors in.....	100%
F_1 hybrids, tumors in	50%
Japanese waltzing mice, tumors in	75%

Although the numbers employed were small, it would appear that the F_1 generation hybrids are, with respect to their reaction to implants of both these tumors, in closer agreement with the Japanese waltzing than with the common parent.

The same author has, on investigating the reaction of

Japanese mice, tame mice and their hybrids to implants of carcinoma (J. w. A.) of the Japanese mouse, obtained results which may be tabulated as follows, the plus sign indicating successful, the negative sign unsuccessful implantation:

CARCINOMA J. W. A.

Japanese waltzing mice	142 +	3 —
Common mice	0 +	48 —
F ₁ hybrids	69 +	1 —
F ₂ hybrids	0 +	54 —
F ₃ hybrids	0 +	16 —

The conclusion, based on these results, that susceptibility to the waltzing mouse tumor was inherited, but neither as a Mendelian unit character nor in accordance with any other hypothesis of inheritance, appeared justifiable on the ground of the complete disappearance of susceptibility in the second (F₂) and third (F₃) filial generations.

The subsequent occurrence of a positive or susceptible hybrid animal of the second filial generation is of importance as indicating the occasional reappearance of susceptibility in subsequent generations (Tyzzer, 1915). A larger series of experiments has thus been planned in order to investigate further the type of inheritance underlying the unusual results obtained in this earlier work.

The material employed in these experiments will now be considered in detail, and later on the results obtained will be discussed and certain conclusions drawn as to the nature of the mode of inheritance here manifested.

MATERIAL.

A single tumor, a carcinoma derived from the Japanese waltzing mouse and designated as tumor J. w. A., has been employed in all the experiments presented in this paper. The histological characteristics and biological behavior of this tumor on transplantation have been discussed in previous articles (see bibliography).

The mice employed consist of Japanese waltzing mice, two distinct stocks of common mice, and hybrids obtained by

cross-breeding the first named variety with the two latter stocks.

1. Japanese waltzing mice are designated in the charts as J. w. The race from which the animals used in the experiments were derived is one that has been inbred for at least six years without any addition of new individuals from outside the stock. The result must therefore have been to produce a race of great uniformity with respect to whatever inheritable factors it may possess. This tendency has also been intensified by the selection of individuals with the smallest amount of black, and, since the amount of black formed depends largely upon hereditary factors, such selection will serve to decrease the number of animals used as parents, thus leading to closer relationships and a greater degree of inbreeding than would result without it. The homogeneity is further shown by the uniformity of their reaction to their tumors. Both carcinoma J. w. A. and sarcomas J. w. B. and J. w. G. have from the first grown on implantation in practically one hundred per cent of this stock. These results are in contrast with those obtained from the implantation of tumors in less inbred stocks. For example, a carcinoma of the stomach, which originated in a laboratory stock of wild mice, grew when implanted in four of eleven, but on the second transfer failed to grow in twelve mice of the same stock. The uncertainty of the results in the experimental inoculation of the tumors of tame mice is a matter of common knowledge. Although the results obtained depend in part on the character of the individual tumor, this is to be regarded as a fairly constant factor, at least at any given transfer, so that, presuming a satisfactory inoculation technic, constant uniformity in the growth of tumor implants furnishes strong evidence of racial homogeneity.

2. Brown agouti stock.—This stock originated from a pen of brown agouti animals which was set aside from the general experiments in color inheritance and inbred since 1909 at the Bussey Institution. This pen of mice contained in all probability several unrelated animals, as no particular

effort was made to pick out closely related individuals. The animals are not always brown agouti in color, but they have been continuously inbred so that they may at least be considered as closely related. Mice from this stock were used in a series of five experiments, A 1 to A 5 inclusive, and will be designated as Br. Ag.

3. Dilute brown stock. — This is the most homogeneous stock of common mice that has been bred at the laboratory of the Bussey Institution. All the present animals are direct descendants of a single pair of closely related, homozygous, dilute brown (silver fawn) mice obtained in the spring of 1909. From the start the stock has been kept free from any out-cross and has therefore an unbroken stretch of more than twenty generations of inbreeding. The mice comprising this race are very strong, healthy, fertile animals, and show not the slightest trace of any harmful results from the continuous inbreeding which has been carried on. Mice of this race are the tame mice used in the experiments B 1 to B 3 inclusive. They are designated as d. Br. in this paper.

It will be seen, therefore, that the stocks used are genetically favorable for obtaining uniform and reliable experimental results. It seems important to emphasize this phase of the work, for if mixed or relatively impure races are used, variable and inconclusive results are almost certain to be obtained. We feel that the material used is of sufficient constancy and definiteness to lend strength to any experimental results obtained in the study of its hereditary behavior.

Each animal born has been given a distinguishing mark and recorded so that accurate pedigrees are available for all the stock used. In order to emphasize the individual as the important unit, a longer time and more care have been necessary than would have been the case if the race had been the unit chosen for study. The general method of recording is that employed at the Bussey Institution in the experiments in color inheritance already reported by one of the writers (Little, 1913). While the data is here presented for the most part by generations, particular emphasis has

been put on keeping as accurate a record as possible for each individual (see appendix for individual records of tumor growth).

Beginning with fourteen days after inoculation, weekly observations have been made and the approximate size of the tumor has been sketched on the card of each individual animal in all the experiments. It is on these records that the conclusions concerning the comparative growth of the tumor in the various generations are based.

KEY TO GENERATIONS.

- Br. Ag Brown agouti, pure stock.
 d. Br. Dilute brown, pure stock.
 J. w. Japanese waltzing, pure stock.
 F₁ First hybrid generation.
 F₂ Second hybrid generation produced by breeding together F₁ animals.
 F₃ Third hybrid generation, produced by breeding together F₂ animals.
 (F₁ x J. w.)¹ First generation back-cross, produced by breeding F₁ animals with pure Japanese waltzing mice.
 (F₁ x J. w.)² Second generation back-cross of F₁ animals with pure Japanese, produced by breeding together the first back-cross animals (F₁ x J. w.)¹.
 (F₁ x Br. Ag.)¹ or
 (F₁ x d. Br.)¹ First generation back-cross, produced by breeding F₁ animals with pure tame races, Br. Ag., or d. Br., as the case may be.
 (F₁ x Br. Ag.)² or
 (F₁ x d. Br.)² Second generation back-cross of F₁ animals with the pure tame races, produced by breeding together animals of the first generation back-cross.

The method of charting is that which has been extensively employed in communications from the Imperial Cancer Research Fund and elsewhere.

The inherited characters which are under consideration in the present investigation are susceptibility and non-susceptibility to carcinoma J. w. A. of the Japanese waltzing mouse. Susceptibility consists of the ability of the inoculated animal to provide a favorable soil for the tumor's growth. This is dependent on the absence of any antagonistic reaction of

the tissues and on a growth of stroma and blood vessels sufficient for the tumor's support and growth. The latter would necessarily be found in the individual in which the tumor originated and would also be expected in closely related individuals. Non-susceptibility is not, however, identical with immunity to implanted tumor, but consists of an ability to develop immunity. The tumor for a period of six or seven days develops as well in the non-susceptible as in the susceptible mice, but this is followed in the former by the appearance of an immunity which is evidently brought about by the formation of a substance or substances that in the presence of living implanted tumor produces injury and inflammation around the latter, so that it eventually becomes isolated from the healthy host tissue necessary for its support. Non-susceptibility would naturally be expected to the implantation of tumor cells of alien races or varieties. It has been repeatedly shown that young animals are in general more favorable for the development of implanted tumors and react more uniformly. Age, therefore, must be taken into account, and is thus given for each animal in the following experiments. Murphy has found that the embryo even of a foreign species will provide the necessary conditions for the growth of tumors until the age at which the spleen and lymphoid organs appear. He also finds that by destroying a large part of the lymphoid tissue by radiation susceptibility to tumor implants is thereby increased. In the present experiments, although some relatively young animals were tested, all were at an age when both spleen and lymph nodes were well developed. In the consideration of susceptibility the comparative rate of growth may be taken into account. The significance of differences in this respect is not clear, since they may depend on modifying rather than on essential conditions. However, the late resorption of tumors, which have developed well and attained considerable size, indicates an effective though delayed immunity reaction, and as such is to be regarded as evidence of non-susceptibility. In fact, a much more pronounced reaction is necessary to destroy a well-developed tumor than a small implant.

EXPERIMENTS.

In certain of the following eight experiments a single tumor was sufficient to inoculate the entire number of mice, in others it was necessary to use two or more tumors to provide sufficient living tumor tissue. In order to obtain adequate control in the latter case, the animals were so grouped that a representative number of each class were inoculated with each tumor. The trochar method of implantation was employed and the inoculation was done as rapidly and with as uniform technic as possible. The dosage was also made as uniform as practicable by this method and the tissue was selected with the view of implanting living tumor in every instance. The groups which previous experience had shown to be probably non-susceptible were inoculated first while the susceptible groups were done last, and the animals were numbered in the order of their inoculation.

The number, class, and age of each mouse, as well as the result of the implantation, will be found in the appended charts, in which the tumors are represented at approximately one-seventh their actual diameter.

EXPERIMENT A I (J. w. A.).

Sept. 3, 1914. — Two J. w. A. tumors which had been growing for thirty-one days in Japanese waltzing mice No. 4058 and No. 4066 were used to inoculate the following seven groups of mice subcutaneously on the right side :

Eleven F_2 hybrids.

Twelve F_3 hybrids.

Eleven back-cross ($F_1 \times \text{Br. Ag.}$)¹ hybrids.

Eleven brown agouti (Br. Ag.) stock mice.

Twelve back-cross ($F_1 \times \text{J. w.}$)¹ hybrids.

Eleven F_1 hybrids.

Eleven Japanese waltzing (J. w.) mice.

On account of the rapid growth of the tumors in the back-cross ($F_1 \times \text{J. w.}$)¹ and the F_1 hybrids, these were killed five weeks after inoculation. The Japanese waltzing mice survived, some for a longer, some for a shorter time. The negative animals of the other groups are charted for seven weeks, although they have since been under observation.

EXPERIMENT A 2 (J. w. A.).

Sept. 17, 1914. — A single J. w. A. tumor which had grown for forty-five days in J. w. mouse No. 4067 and weighed 1,700 grams was used to inoculate one hundred and thirty-one mice classed as follows:

Fifty F_2 hybrids.

Thirty-seven back-cross ($F_1 \times \text{Br. Ag.}$)¹ hybrids.

Six brown agouti (Br. Ag.) stock mice.

Twelve back-cross ($F_1 \times \text{J. w.}$)¹ hybrids.

Six F_1 hybrids.

Twelve Japanese waltzing (J. w.) mice.

Eight second generation ($F_1 \times \text{Br. Ag.}$)² back-cross hybrids.

Each mouse received approximately 13 milligrams of tumor tissue. An F_2 hybrid (No. 4211) produced a slowly growing tumor which failing to retrogress killed the animal nearly fourteen weeks after inoculation.

EXPERIMENT A 3 (J. w. A.).

Oct. 13, 1914. — Bits of the tumor which had grown forty days in J. w. mouse No. 4163 were used to inoculate one hundred and eighteen mice; average dose 10 milligrams.

Twenty-seven F_2 hybrids.

Five F_3 hybrids.

Sixteen back-cross ($F_1 \times \text{Br. Ag.}$)¹ hybrids.

Forty-one brown agouti (Br. Ag.) stock mice.

Six back-cross ($F_1 \times \text{J. w.}$)¹ hybrids.

Eleven F_1 hybrids.

Three Japanese waltzing (J. w.) mice.

Five second generation ($F_1 \times \text{Br. Ag.}$)² back-cross hybrids.

Four ($F_1 \times F_2$)¹ hybrids.

A second exception to the usual results was obtained in this experiment. In an F_2 hybrid (No. 4392) a tumor developed which grew slowly for eight weeks, when the animal disappeared, possibly from being eaten by other mice in the same cage. Of four progeny of an F_1 mated with an F_2 hybrid, one developed a progressively growing tumor.

EXPERIMENT A 4 (J. w. A.).

Oct. 27, 1914. — A tumor weighing 4.010 grams which had grown fifty-four days in J. w. mouse No. 4121 was used to inoculate twenty-seven mice; average dose 30 milligrams.

Eleven F_2 hybrids.

Three F_3 hybrids.

Four F_1 hybrids.

Six Japanese waltzing (J. w.) mice.

Three ($F_1 \times F_2$)¹ hybrids.

Two of the last group survived and one of these developed a tumor.

EXPERIMENT A 5 (J. w. A.).

Jan. 15, 1915. — A tumor after growing thirty-six days in J. w. mouse No. 4393 was used to inoculate the following one hundred and sixteen mice:

Thirty-three F_2 hybrids.

Seven F_3 hybrids.

Seven back-cross (F_1 x Br. Ag.)¹ hybrids.

Twenty-six back-cross (F_1 x J. w.)¹ hybrids.

Ten F_1 hybrids.

Six Japanese waltzing (J. w.) mice.

Nineteen second generation (F_1 x Br. Ag.)² back-cross hybrids.

Three (F_1 x F_2)¹ hybrids.

Five second generation (F_1 x J. w.)² back-cross hybrids.

Only one in the five animals of the last group developed a tumor, although derived from susceptible parents.

EXPERIMENT B 1 (J. w. A.).

Sept. 25, 1914. — A single J. w. A. tumor weighing 1.100 grams, after growing fifty-three days in a J. w. mouse, No. 4056, was used to inoculate eighty-four mice of the following classes:

Twenty-six F_2 hybrids.

Five F_3 hybrids.

Twenty-one dilute brown stock mice.

Twenty-two F_1 hybrids.

Ten Japanese waltzing mice.

This experiment yielded no unusual result.

EXPERIMENT B 2 (J. w. A.).

Oct. 27, 1914. — A tumor which had grown to .834 gram in J. w. mouse No. 4121 in fifty-four days was inoculated subcutaneously in the following forty-eight mice; average dose 11 milligrams.

Twenty-three F_2 hybrids.

Nine back-cross (F_1 x d. Br.)¹ hybrids.

Ten dilute brown stock mice.

Six Japanese waltzing mice.

In one F_2 hybrid (No. 4551) a small nodule appeared, which did not increase in size between the second and the sixth week after implantation, but subsequently grew to large size. The mouse was killed seventy-two days after inoculation and the tumor employed in the following experiment.

EXPERIMENT B 3 (J. w. A.).

Jan. 7, 1915. — The following eighty-nine mice were inoculated subcutaneously on the right side with J. w. A. tumor which had been growing twenty-six days

in J. w. mouse No. 4594 and similarly on the left side with tumor J. w. A. which had been growing seventy-two days in an F_2 hybrid (No. 4551).

Thirty-five F_2 hybrids.

Eleven F_3 hybrids.

Eleven dilute brown stock mice.

Nineteen back-cross ($F_1 \times J. w.$)¹ hybrids.

Five second generation ($F_1 \times d. Br.$)² back-cross hybrids.

Eight Japanese waltzing mice.

The object of inoculating a J. w. A. tumor which had been successfully transplanted to an F_2 hybrid was to determine whether adaptation to a somewhat unfavorable host would influence its subsequent behavior. There was, however, no evidence of adaptation on the part of the tumor, for it showed no more growth in the non-susceptible classes of mice than did the tumor from the J. w. mouse.

If now the results of these experiments are analyzed (see appended charts), it will be seen that the two tame races do not show any marked differences in their reaction to the tumor either as pure stocks or in their hybrids with the Japanese race.

COMBINED RESULTS OF EIGHT EXPERIMENTS WITH CARCINOMA J. w. A.

Class of Mouse.	Number Inoculated.	Susceptibility.	
		+	-
J. w.....	58	58	0
Com.....	99	0	99
F_1	62	61	1
F_2	183	3	180
F_3	38	0	38
($F_1 \times J. w.$) ¹	63	63	0
($F_1 \times J. w.$) ²	5	1	4
($F_1 \times Com.$) ¹	78	0	78
($F_1 \times Com.$) ²	34	0	34
($F_1 \times F_2$) ¹	9	2	7
Total.....	629*		

* Sixty-three additional animals died too soon after inoculation to be included.

It will, therefore, be convenient to tabulate together the data obtained in the A and B series of experiments. If this is done, it will be seen that the difference between the common races and the Japanese race is absolute. The ninety-nine mice of the common stock who lived for a sufficient period after inoculation to provide a critical test as to their reaction to the tumor transplant, all failed to grow the tumor. On the other hand, the forty-eight Japanese mice were all of them positive, having well defined tumors which showed a steady growth (see appendix).

Of the F_1 hybrids obtained from cross-breeding common and Japanese mice, sixty-one out of sixty-two were susceptible. It is not known whether the one negative F_1 animal was a true exception or not, since no reinoculation was attempted. It is quite possible that its negative behavior may have been due to some unavoidable circumstance of technic in inoculation.

The F_2 generation hybrids show in contrast to the 98.3 per cent susceptibility of the F_1 generation a remarkable decrease in the number of susceptible animals. Only three out of one hundred and eighty-three recorded grew the tumor. This is approximately 1.6 per cent positive. The number used is considerable. Both races of common mice have susceptible F_2 animals among their hybrid offspring.

The ancestry of the two susceptible F_2 mice in the A series of experiments (Br. Ag. stock) is of interest. The first one, mouse No. 4211 (male 656 stock number), was out of a bi-maternal litter; two sisters, 104 and 106, having littered together. The father of both litters was 57. The second susceptible F_2 mouse, No. 4392 (female 777 stock number), came from a later mating of female 106 by male 57. She was then certainly a half sister and possibly a full sister of mouse No. 4211, the first susceptible animal obtained in these experiments. As to what significance, if any, is to be attached to this fact of close relationship, we are at present in doubt. It is peculiar that two in thirteen mice derived from these F_1 hybrids were positive, whereas one hundred and one of other parentage were negative. If

any marked tendency of this sort is met with, it will be necessary to consider something more than chance in the distribution of susceptibility in the F_2 generation as a whole. The fact that a susceptible F_2 mouse has occurred in the entirely unrelated B series makes it certain, however, that it is a phenomenon of general applicability and that we are not dealing with a peculiarity restricted to a single family of the brown agouti race.

The number of F_3 animals is unfortunately not very large, only thirty-eight being thus far tested. All these have proved negative. Positive animals will probably be obtained in this generation when larger numbers are raised.

The reaction to the implantation of carcinoma J. w. A. in the back-cross generations is of considerable interest. The young produced by crossing the F_1 hybrids with pure Japanese mice are very difficult to raise. The young animals are delicate and are markedly susceptible to disease. Of course, the fact that this cross should produce a generation fifty per cent of which are waltzers undoubtedly accounts in part for the high mortality, as waltzing mice are notoriously difficult to rear. However, even the non-waltzers in this particular generation appear to be less vigorous, and it is evident that the Japanese race is one which is not well fitted to withstand conditions under which common mice thrive. It appears probable that these back-cross animals, really three-fourths blood Japanese, inherit to a considerable degree the constitution of the Japanese race. They furthermore simulate more closely the Japanese mice in their reaction to the tumor transplant, one hundred per cent of them being positive in a total of sixty-three animals.

Only five young have been raised from these back-cross ($F_1 \times J. w.$)¹ mice. These have, however, shown a markedly different degree of vigor and a different behavior towards the tumor implant. They are normal-appearing, healthy mice, easily raised after weaning, and on inoculation four out of five have been found non-susceptible to the implant.

The parallel back-crosses between F_1 generation hybrids and common mice have produced uniformly vigorous, healthy

mice. Seventy-eight of the first generation of this back-cross were inoculated with negative result in all. Thirty-four of the second generation produced by breeding the first generation back-cross animals *inter se* were all non-susceptible.

The one remaining cross, that of F_1 generation mice, bred to their F_2 offspring, has only given a very small number of young. It tends to show, however, that there is an increased liability to tumor growth in these animals as compared with those of the F_2 and F_3 generations. In the nine animals inoculated there were two which grew tumors.

The biological character of the tumor through its continued growth in an F_2 hybrid has not been appreciably modified, for it subsequently reacted on implantation in every respect similar to parallel implants of tumor taken from the Japanese waltzing mouse (see Experiment B 3). Adaptation of the tumor, therefore, is evidently of slight importance in the interpretation of the data at hand, and variation in the host rather than in the tumor appears to determine the results.

The total number of mice (six hundred and twenty-nine) on which our conclusions are based represent those of the six hundred and ninety-two inoculated which survived long enough to give a critical test as to their reaction to the tumor implant. The growth charts given in the appendix will show the occurrence of the sixty-three animals which died so soon after inoculation that they could not be considered as a critical part of the experiment.

DISCUSSION.

It is the common experience of investigators of the inheritance of size characters in plants and animals that in a cross involving races of distinct sizes the first hybrid generation shows a condition intermediate between the parents. Examples of this type of result have been recorded by Castle with respect to the ear length of rabbits; by MacDowell in studying the inheritance of body size in rabbits; by East in various size characters of tobacco and of maize; and by many other investigators working with very diverse types of plants

and animals. Whenever in such crosses the F_1 hybrids are bred together they give rise to an F_2 generation in which most, if not all, of the individuals fall between the parent forms in respect to size and are therefore generally considered intermediate. The F_2 animals show usually a distinctly greater degree of variability in respect to size than do the F_1 hybrids, and in some cases individuals may be produced even more extreme in size than the parent types. Such inheritance has been given by Castle and others the general name of *blending* inheritance, as contrasted with *Mendelian* inheritance.

Whether or not a system of multiple Mendelian factors will prove to be the basis of this apparently blending type of inheritance will not here be considered. It will be sufficient to say that blending inheritance as supported by such evidence is practically the only type which occupies the minds of investigators in conjunction with or in place of Mendelian inheritance.* It is therefore of importance for us to test the applicability of an hypothesis of blending inheritance to the results of the experiments here recorded. If blending inheritance can be eliminated as a possibility, the field will be left just so much the clearer for the consideration of a Mendelian explanation.

Blending inheritance hypothesis.—The first point of importance bearing on this question is that the two parent races used in our experiments differ absolutely from one another with respect to their reaction to implantation of the waltzing mouse tumor. The transplanted tumor grows in approximately one hundred per cent of the inoculated animals of one race, while it grows in none of the other races. Here then is the best possible chance for intermediates to occur when these widely divergent races are crossed. The

* Slye states that as a result of mating an animal having a dominant character with another having a recessive character, offspring are produced some of which are pure dominants, lacking the recessive character, others of which are hybrids having both the dominant and the recessive characters, as may be demonstrated by the appearance of the latter in their offspring. This cannot in any way be correlated with either blending or Mendelian inheritance.

first generation hybrids, however, grow the tumor about as successfully as the positive parent race, *i e.*, in 98.4 per cent. If this is to be regarded as an intermediate result, then on the basis of blending inheritance it would be expected that a distinct majority of the second hybrid generation animals should grow the tumor successfully. This result is not obtained, for only one animal in approximately each sixty (or 1.6 per cent) of the second hybrid generation grows the tumor (see table). In a few others the tumor grows for a time but eventually disappears. This cannot be considered as a blending result, for even if all those animals which presented a greater development of the tumor than the non-susceptible parent stock are taken into account, the results cannot be considered as intermediate. The number of animals showing this condition is small, and we do not find a single instance of it in the F_1 generation, where we should expect the most perfect field for "blending" to occur. Alternative inheritance with respect to tumor susceptibility is clearly operative. In the back-cross generations also clear evidence of a non-blending type of inheritance is seen. Thus, the animals produced by a cross between F_1 and the positive parent are one hundred per cent positive, and those produced from F_1 crossed with the negative parent are all negative. No sign of blending is observed.

The results are markedly distinct in that the hybrid generations present alternative rather than intermediate conditions with respect to the reaction to the implanted tumor. We are unable, therefore, to interpret the results obtained in our experiments on the basis of the hypothesis of blending inheritance.

It then becomes necessary to consider whether the facts of inheritance in this case are in accord with those of the inheritance of color characters and of other Mendelian characters in mice. Are we dealing with some form of Mendelian inheritance which although obscure, because of the complex and hidden nature of the factors involved, is nevertheless based upon the random segregation of germinal units? The adequacy of the Mendelian laws in explaining

the observed facts of color inheritance in mice is well known. It has been the common experience of almost a score of investigators in Europe and in this country that analysis of the phenomena of color inheritance in general has advanced most rapidly through adopting mendelizing unit factors or determinants as the basis for genetic differences between the various color varieties.

Mendelizing factors which are of opposite nature so that they cannot both be contained in a single unfertilized germ cell are termed "allelomorphic." Since individuals or "zygotes" are formed from the fusion of two gametes or germ cells, it follows that in respect to any two allelomorphic factors an individual may be of the following constitution: If A and a are the allelomorphic factors, the individual may be AA, aa or Aa, according to whether it was formed from the union of gametes *like* (AA or aa) or *unlike* (Aa) with respect to the factor in question. The first two combinations (AA or aa) are called "homozygous," being formed from the union of *like* gametes. The last combination (Aa) is "heterozygous," being formed from the fusion of *unlike* gametes.

In such a heterozygote as Aa, if the A character expresses itself to the exclusion of the a character, it is called "dominant," while the a character is called "recessive," and is evident only in individuals which are aa in constitution. Thus, completely colored animals may be either homozygous or heterozygous with respect to the character pigmentation, since the factor for color is dominant; while albinos must always be homozygous, since the factor for albinism is recessive. If the heterozygote is a blend between A and a, dominance is said to be imperfect.

At the present time there are at least seven allelomorphic groups of color factors and one pair of allelomorphic physiological factors known in mice. It is not necessary to go into detailed consideration of the evidence on which the existence of these allelomorphic groups rests. It is sufficient to say that they have each been recognized by more than one investigator (see bibliography). It is not at present

certain whether the members of any one group are the result of the presence of specifically different germinal factors or whether they represent merely distinct grades of activity or different forms of a single factor. For the present purposes it makes little difference which of these conditions actually exists. All that is necessary to consider here is the fact that many independent hereditary units are active in forming the various color varieties of mice.

The terms used in describing the color varieties of mice and the factors involved in their formation are for the most part those used in a previous publication by one of the writers (see Little, 1913).

The groups of allelomorphs in mice are as follows:

- | | |
|--|-----------------------------|
| (1) C — full pigmentation (Y of some authors). | c — complete albinism (y). |
| (2) D — density of pigmentation. | d — dilute pigmentation. |
| (3) S — solid coat (U of some authors). | s — spotted coat (u). |
| (4) F — solid forehead. | f — blaze (white forehead). |
| (5) P — full black and brown eye pigmentation. | p — pink eye reduction. |
| (6) A — agouti coat (G of some authors). | a — non-agouti coat (g). |
| (7) B — black pigmentation. | b — non-black pigmentation. |
| (8) W — non-waltzing. | w — waltzing. |

These groups of allelomorphs have all been proved to be independent of one another in inheritance. This is not very surprising, for Morgan has found in the fruit fly, *Drosophila ampelophila*, that complete independence is to be expected in inheritance unless factors are borne in the same chromosome.

Since the researches of certain investigators in the oögenesis of mice have fixed the number of chromosomes in mice at twenty, we should expect that an equal number of allelomorphs might well be found which were entirely independent in inheritance. Morgan and his pupils have identified and investigated the inheritance of more than fifty-five groups of allelomorphs in *Drosophila*. With this fact in mind, an hypothesis utilizing ten or twenty pairs of mendelizing factors loses much of the fantastic and speculative appearance which it would have presented before their work was recorded.

Single Mendelian factor hypothesis. — The next point to be considered is whether or not a simple Mendelian hypothesis will account for the results of the present series of experiments. It is apparent from the outset that if a single factor underlies susceptibility to the transplantable tumors in mice, this is dominant over non-susceptibility. On this supposition approximately seventy-five per cent of the F_2 generation should be susceptible, whereas only 1.6 per cent of this generation have proved so. The almost complete absence of susceptible animals in this generation suggests that we may possibly be dealing with a reversal of dominance. If this were true we should expect a return of susceptibility, which on reversal would become recessive in at least twenty-five per cent of the F_2 and F_3 generations. The results which we have obtained, therefore, make it necessary to abandon the idea that there is in the stocks with which we have worked a single factor difference to account for susceptibility and non-susceptibility to the transplanted tumor.

Slye has published a report of experiments on the inheritance of the spontaneous tumors of mice. She finds, as have several earlier investigators of this problem (see Tyzzer, 1907; Murray, 1911), that various families show distinctly different hereditary tendencies in their ability to form spontaneous tumors. In the paper referred to Slye states that she is treating cancer as a *unit character*, that she has used methods in testing the inheritance of this character similar to those used in judging the inheritance of such a character as albinism. From her treatment of the subject throughout the paper it is evident that she considers *factor* and *character* as interchangeable or synonymous terms. That such is only rarely the case has been shown by a number of the more recent researches of both plant and animal geneticists.

When one considers that the reactions of mice to a single transplantable tumor are extremely diverse and certainly do not depend upon a single mendelizing factor, it seems hardly conceivable that the origin of the many types of spontaneous tumors in mice will prove to be dependent on a single

factor, or that they can all of them be grouped together as a single unit character.

As to Dr. Slye's statement that the problems of the inheritance of cancer cannot be solved by investigations with transplantable tumors, it is hoped that as the study of growth regulation is an important phase of tumor investigation, so the study of the inheritance of factors preventing or allowing abnormal growth may later on assist in the solution of the problem of cancer inheritance.

Since the single mendelizing unit, as well as the blending inheritance hypothesis, fails to explain the present results, the inheritance of susceptibility to transplanted tumor on the basis of multiple factors showing Mendelian inheritance may next be considered. Unless this applies, we shall be forced to admit that the facts are inexplicable on any present theory of inheritance.

The multiple factor hypothesis. — This may apply in several different ways as follows:

Cases have been reported by several investigators in which a given character may be produced by the action of any one of several independent mendelizing factors. One of the best known examples of such a condition is that reported by Nilsson-Ehle in wheat, where red color is produced by the action of any or all of three independent factors, which may be designated A, B and C, respectively. When all of the three are lacking, and only when this is the case, a white wheat is produced. The wheats possessing either A, B or C are red, and we have, therefore, in the F_2 generation from a cross between red (ABC) and white (abc) wheat a ratio of sixty-three red to one white individual, as follows:

27....	ABC	
9....	ABc	
9....	AbC	
9....	aBC	
3....	Abc	
3....	aBc	
3....	abC	
— — —		Sixty-three red.
1....	abc	
— — —		One white.

In this case all the individuals having the dominant character, red, are more or less similar, so that they have been classified under one descriptive term irrespective of whether the color is based on the presence of one, two, or three of the necessary factors. In raising only a small number of F_2 plants it is quite probable that the one white individual expected in approximately every sixty-four of this generation might not be obtained. There thus is no *a priori* ground for not expecting to find characters depending for their manifestation on one of four, five or n factors, and having an alternative form produced only when all four, five or n factors were inactive. The fact of interest in connection with the present work is that many factors, or better perhaps, "absences of factors," are in some instances necessary in order to produce a given character. In the example given above the application of this principle would extend only to recessive characters.

Attention may now be called to an allied yet distinct condition. The possibility of a dominant character dependent for its manifestation upon the simultaneous presence, in either a homozygous or heterozygous condition, of several factors may now be considered. Such a character will appear to be dominant instead of recessive, yet the principle of multiple factors underlying its formation is entirely Mendelian. A well-known example of a dominant character dependent upon the simultaneous presence of two independent mendelizing factors is that of color as opposed to albinism in sweet peas (Bateson, Punnett). Here the two factors, which we may call A and B , must both be present in either a single or double "dose," that is, either in a heterozygous or homozygous condition, in order for any color to be formed. The experimental result is that when (colored) $AABB$ plants are crossed with $aabb$ (white) individuals all the F_1 generation is colored $AaBb$, the single "dose" of A and B together being sufficient to produce

color. If F_2 is raised, it will consist of the following types:

1....	AABB	
2....	AaBB	
2....	AABb	
4....	AaBb	
<hr/>		Nine colored.
1....	AAbb	
2....	Aabb	
1....	aaBB	
2....	aaBb	
1....	aabb	
<hr/>		Seven white.

Only two somatic types, colored and white, will be formed. These will, however, bear to one another a 9:7 ratio instead of a 3:1 ratio, as is the case in F_2 from a cross involving only one factor as the basis of the character under observation.

This general line of reasoning may be carried further and a character formulated which depends upon the simultaneous presence of three factors A, B, and C. Here F_2 will consist of twenty-seven individuals with at least one "dose" of A, B, and C (therefore having the character in question) to thirty-seven individuals lacking one or more of the three factors A, B, and C (therefore lacking the character in question). We thus see that when one pair of factors is involved, the F_2 ratio is three individuals with the character to one without the character. When two pairs of factors are involved, the ratio in F_2 becomes nine with, to seven without, the character, or 1.28 to 1. When three pairs of factors are involved, the balance shifts still further, so that there are more individuals (37:27) without the character in F_2 than with it, the ratio being 1 to 1.37. As the number of pairs of factors involved increases, the ratio grows more and more unequal. Thus, with ten pairs of factors the ratio is 1 to 17.7, and with twenty pairs of factors a ratio of 1 to 314.3 (see Little, 1914).

The objection may be raised that no case is known in actual experimentation, with the exception of that of

sweet peas, which fits this especial phase of Mendelian inheritance. We believe, however, that the method of inheritance of certain color characters may be considered comparable to that of the tumor susceptibility which we have studied, and that in both cases the type of inheritance involved is identical. Eight pairs of allelomorphic characters have already been utilized in certain of our experiments with mice. While a single cross involving all eight pairs has never been made, it is nevertheless fairly certain that they are independent in inheritance and would give the following results:

If a wild mouse, intense, dark-eyed, black, agouti, and solid coated, is crossed with a waltzing albino potentially dilute, pink-eyed, brown, non-agouti, with two types of spotting, the offspring of the F_1 generation should all resemble the wild parent in color and habit.

The F_2 generation should yield two hundred and fifty-six color varieties (see Appendix B). Of these, sixty-four will be albinos and therefore identical in appearance though genetically distinct. Of the one hundred and ninety-two remaining varieties only one would exactly reproduce the F_1 hybrid type characteristic of the wild grandparent. The other one hundred and ninety-one would show variations of many sorts, but would all be distinguishable by breeding tests from one another. Thus, only one variety in the two hundred and fifty-six which it is possible to produce will exactly resemble the wild grandparent in its color and habit. As will be seen from the data given in Appendix B, however, the number of individuals in this one class is three times as great as that in any other one variety, and will bear to all other F_2 forms together approximately a 1 to 10 relationship. If instead of eight factors we were dealing with ten, the ratio would be 1 to 17.7.

Most of the color varieties which have been studied are distinguishable from one another somatically. If, however, the characters in question had to do with susceptibility to implanted tumor or some other physiological peculiarity, less evident somatically than pigment formation, we should

expect that a majority of the F_2 varieties would be indistinguishable from one another. This would lead to the lumping together of the forms of F_2 which do not possess at least a single dose of all the factors necessary for tumor growth under the general head of "non-susceptible." That this is the sole difference between the inheritance of visible characters and the inheritance of susceptibility to transplantable tumors is strongly indicated by the exact agreement of the two when all color varieties not resembling the wild mouse are lumped together. The results of the present experiments are thus most readily explained on the basis of the multiple Mendelian factor hypothesis. Aside from this we have no other known type of inheritance which applies to the data obtained.

No signs of linkage of the factors for tumor growth with the known color factors have been observed in our experiments. Since, however, the material had not been chosen with any idea of testing this particular problem it is not surprising that signs of linkage have not been observed. The possibility that some degree of linkage between factors affecting tumor growth and color factors may exist must therefore constantly be borne in mind. The object of the present investigation is, however, to show that many factors are involved in determining the reaction of a given animal to the tumor J. w. A. and that these factors are independent of each other.

In the following chart the plus sign indicates the presence of all the factors necessary for the character in question, *i.e.*, in one series the "wild" coat character, in the other "susceptibility" to the inoculated tumor. On the other hand, the minus sign indicates the absence of some of the factors necessary for the character in question. The five generations in which large numbers were obtained in the tumor inheritance work are included in the chart. The results obtained in other generations are omitted because the numbers thus far tested appeared too small to afford critical evidence for the

similarity of the two cases. In so far as they go, however, they are in accordance with the other results.

Color Characters.	Generation.	Tumor Growth Characters.
(a) Wild house mouse (agouti) +	Parent.	Japanese waltzing mouse (susceptible) +
(b) Albino -		Common mice (immune). -
All of wild house mouse color +	F ₁	All susceptible +
Only an occasional individual +	F ₂	Only an occasional individual +
Great majority -		Great majority -
All of wild mouse color.... +	Back cross F ₁ with + parent.	All susceptible +
Theoretically, if there were more than ten factors, one in several thousand would be like wild parent; all the rest would be unlike in some respect.	Back cross F ₁ with - parent.	None have proven susceptible; all are..... - Theory applicable to color factors applies here.

By comparison the similarity in the inheritance of the two types of character is remarkable.

Before testing the application of our hypothesis to the work already recorded by other investigators, it may be well to point out two lines of further investigation which we hope to pursue in order to test definitely the applicability of the theory of multiple factors to the case of transplantable tumors.

First, detailed matings and tests of various lines of F₃ animals will be followed up carefully. Such tests should reveal the presence of various strains differing from one another in the factors for tumor growth which they possess. These differences will be manifested by corresponding differences in the ratios of susceptible to non-susceptible animals

in the offspring of the various lines. It should be possible to isolate strains of F_2 or F_3 hybrids, giving a much more easily observed ratio than the present one of one to sixty in the F_2 generation. As the lower ratios will also mean that the animals involved have only a few factors differing from the Japanese, the effort to obtain strains having low ratios will have a double object.

Second, the offspring obtained by breeding together the young from the cross between Japanese and F_1 hybrids should give a larger number of susceptible animals than the straight F_2 or F_3 generation. This increase should be in a definite ratio, according to the number of factors involved, and will, therefore, be open to experimental tests. If confirmatory results are obtained from these two sources and distinct advances are made towards the isolation of a strain of hybrids differing by five or less factors from the Japanese, it may fairly be claimed that the correctness of the hypothesis will have been established.

Until further experiments are made we should think of the growth of the transplanted tumor as depending not upon any determined number of factors, but rather upon a certain factor complex which is found in essentially all the animals of the Japanese waltzing race. Conversely we may think of the common (brown agouti or dilute brown) race as possessing a different factor complex from that of the Japanese race.

The complex of the tame race is capable of producing immunity to the tumor in the presence of part of the Japanese factor complex. If, however, all the members of the Japanese factor complex are represented, as in F_1 , tumor growth is produced. The common race has a factor complex which by itself produces an environment unfavorable to tumor growth, while the opposite is true of the Japanese race. The hybrids of the first generation contain a sample of both complexes, and here the elements favorable to tumor growth furnished by that derived from the Japanese parent are sufficient to overcome any tendency to immunity which

that derived from the common parent might tend to produce.

When, however, the gametes are formed by the F_1 animals to produce the F_2 generation, the factors in the Japanese complex being mendelizing units are distributed in these gametes by the law of chance, and accordingly only one gamete in a great many will contain all the members of the Japanese factor complex. The fact that a great majority of the F_2 animals are non-susceptible to the inoculated tumor indicates that when only part of the members of the Japanese complex are present, continued tumor growth is impossible. The fact also that in certain F_2 animals a temporary growth of the tumor takes place, to be followed in turn by complete retrogression of the implant, may be explained on the basis that such animals represent a factor complex which contains most but not quite all the elements or factors found in the pure Japanese race. This temporary growth shows further that if the hypothesis of multiple factors is correct, certain of the factors probably reach their expression as active forces on tumor growth at different periods during the development of the individual. This is at least true of all mendelizing characters which become visibly manifested.

Although dominance of "susceptibility" in the F_1 generation suggests the presence in the Japanese race of a considerable number of epistatic* factors, such is in all probability not the case. The appearance of "susceptibility" as a dominant does not necessitate the addition of substances lacking in the tame race. In fact, quite the opposite is probably true. Thus, if we suppose the "Japanese" complex to be made up of $A^J B^J C^J D^J \dots \dots X^J$, and the "tame" complex of a number of factors $A^T B^T C^T D^T \dots \dots X^T$, then the one condition necessary for susceptibility to appear is the presence of at least a single representation of all the factors characteristic of the Japanese complex, as in the F_1 hybrid $A^J A^T B^J B^T C^J C^T D^J D^T \dots \dots X^J X^T$. Various recombinations of these factors in the F_2 generation fail to

* Genetic not medical terminology.

produce susceptibility unless at least a single dose of all the factors in the Japanese complex is present. Thus, if a certain F_2 animal was of the formula $A^J A^T B^J B^T C^T C^T D^J D^T \dots X^J X^T$, it might be non-susceptible since it did not possess even a single dose of the C^J factor, even though all the other factors in the Japanese complex were represented.

Application of the Multiple Factor Hypothesis to the Work of Other Investigators.

Cuénot and Mercier have published a report on the results of certain experiments which they have carried on to determine the nature of the inheritance of susceptibility and immunity to an inoculable tumor of common, *i.e.*, non-waltzing mice.

They conclude that susceptibility and non-susceptibility are not Mendelian characters, and that neither hypotheses of one, two or n factors will explain the facts in an exact manner. It should, however, be remembered that when this was written by them the modern methods of utilizing multiple factors were to all intents and purposes unknown.

So far as their results are given, they can be satisfactorily explained on the hypothesis which we have advanced. They themselves feel that the character inherited is a certain racial percentage of "takes." This type of explanation, while it is similar to that adopted by Jennings, in explaining the inheritance of size differences in *Paramecium*, and while it accounts for Cuénot's and Mercier's results, fails to fit the facts observed in Tyzzer's earlier work or in the experiments here recorded. The idea of "genotypes" or races differing from one another in their complex of hereditary factors is the most applicable part of their explanation. It is, of course, this idea of "genotypes" that underlies the modern theories of multiple factors and of "pure lines."

The hypothesis, however, that there are races of mice which differ from one another constantly in the percentages of the "positive" and "negative" animals which they contain is not supported, in the form in which Cuénot and Mercier have advanced it, by the writers' experiments.

The essential difference between their hypothesis and that which we advance is the following: On their supposition a genotype when isolated will consist of animals all of which possess the ability to produce a certain fixed percentage of positive or susceptible animals among their progeny. The animals contained in this genotype will continue indefinitely to produce animals as descendants all of which possess the same ability as they did. It is as though we had between the conditions of one hundred per cent positive and zero per cent positive a number of permanent intermediate percentages which represented the centers of variation of a number of genotypes.

We may now imagine that one of these genotypes has a characteristic percentage of eighty per cent positive and another of twenty per cent positive animals. These two genotypes having been carefully isolated, the breeding of either positive or negative animals would not result in permanently modifying the percentage of takes characteristic for the genotype. No animal transmitting either a higher or a lower percentage of "positives" than that characterizing their particular genotype could be obtained without the appearance of a mutation. This naturally is a possibility in any material, but is a phenomenon of so infrequent occurrence that it may be left out of consideration in the present case.

On our hypothesis, wherever a positive animal appears it means that this animal contains at least a single representation of the factor complex of the (Japanese) one hundred per cent positive race. This means that this animal is at least as near to the make-up of the one hundred per cent positive parent as are the F_1 hybrids between common and Japanese mice, even though it may occur in a generation giving only 1.6 per cent positive or even less.

Moreover, on Cuénot's and Mercier's hypothesis that, when we have two races apparently the extremes of a plus and minus variability in the degree of susceptibility, we should expect an intermediate and blending inheritance when these two forms are crossed, but such a result was not

obtained. It is scarcely conceivable that the factors underlying susceptibility and non-susceptibility to inoculated tumors in the races of common mice used by the other investigators are fundamentally different from those found in the common mice which we used. It is much more probable that they are similar factors but present in our races in a degree of homozygosis approaching a genotypical condition; while in their material the factors were almost certainly more unevenly distributed, approximating the condition characteristic of mixed populations in general. If, therefore, the factors in the various cases are fundamentally the same, any general hypothesis explaining the facts of inheritance of part of the experimental results should be applicable to the results as a whole. This we believe to be true of the hypothesis which we have advanced.

In addition to the work already referred to, Loeb and Fleischer have carried on a series of investigations on the factors underlying the hereditary susceptibility of mice to a transplantable carcinoma.

The material which they used was in several ways distinctly different from that afforded by the tame and Japanese races and their hybrids. It was, however, of such a nature as to furnish an interesting line of support to the evidence which we have been able to obtain.

Loeb and Fleischer used one race of American mice and two European races, obtained by distinct importations, as their parent stocks. They found by tests reaching over several generations that the percentage of American mice to grow the inoculated tumor was eighty-four, while those of the European races I and II were twenty-three and three per cent respectively.

They next proceeded to cross the American race with each of the two European races. They found that F_1 from American \times European I gave sixty-eight per cent susceptible, while F_1 from American \times European II gave one hundred per cent susceptible. F_2 from the American \times European I gave thirty per cent susceptible and F_2 from American \times European II gave twenty-six per cent susceptible.

It will at once be noticed that there are certain marked differences between the results above mentioned and those obtained by Tyzzer, 1909, and by the writers. In the first place, in the experiments of Loeb and Fleischer the F_1 generation showed a percentage of susceptibility intermediate between the parent races. The F_1 mice obtained from American mice crossed with European race II, which were one hundred per cent susceptible, were in too small a number (14) to establish the susceptibility of this generation. The decrease of the percentage of susceptible animals in F_2 is in accordance with the previous work of Tyzzer and with the present experiments.

These points of difference and of similarity are readily understandable on a Mendelian hypothesis of multiple factors. We have supposed that in the case of the experiments reported in this paper a number of factors are found in the germ cells of one race which are either not present or replaced by allelomorphic factors in the other race. We have further supposed that successful growth of the transplanted tumor depends upon the simultaneous presence in the zygote, at least in a single representation, of a considerable number of factors of the susceptible race.

This hypothesis will apply as well to the experiments of Loeb and Fleischer. The fact that the American race gave in the neighborhood of twenty per cent of its members non-susceptible to the transplantable tumor shows at once that it is not a race homozygous for the susceptibility producing factor complex. The percentages of susceptibility in the two European races show that they both probably have even fewer of the necessary factors in a homozygous state, and that they probably differ from one another as well. Race I, for example, has probably distinctly fewer of these factors in a homozygous condition than has race II.

The cross with the American race produces exactly what might be expected. In the cross of American by European I the F_1 generation is intermediate in its percentage of susceptibility and F_2 shows that the combinations of factors necessary for tumor growth are less common than in the

F₁ generation. This is to be expected on the multiple factor hypothesis. The same applies to the F₁ and F₂ from the American by European II cross.

The character of the F₃ from these crosses may be predicted as follows on the hypothesis of multiple factors: If a large number of F₂ animals, mated inter se at random, are used to produce the F₃ generation, the percentage of susceptibility in the F₃ generation should be the same as in F₂. This condition of random mating, producing large numbers, is most nearly realized in the F₃ generation of the cross between American and European I. The one hundred and twenty-two animals comprising this generation show a percentage of susceptibility of twenty-four as compared with thirty per cent in the F₂ generation of the same cross. In the F₃ generation of the American by European II cross the percentage of susceptibility in sixty-six animals is only two as compared with twenty-six per cent in the F₂ of this cross. This difference is probably at least partly due to the relatively small numbers of young raised in that particular cross.

The behavior of the back cross generations tested by these authors is also amply in accord with this hypothesis of multiple factors, and it appears that this set of results, which almost completely parallel the results of the usual "size inheritance" crosses, offers extremely valuable evidence in showing the close relationship of an apparently blending inheritance and an obviously alternative inheritance of the characters on which susceptibility to inoculable tumors are based.

The results of the present series as well as of previous experiments may be explained by the hypothesis that susceptibility to a transplantable tumor is based on a factor complex, or on the coexistence of a number of inherited factors in the individual. These factors, even when in only a single representation (or half dose), as they must occur in the first filial generation of hybrids, are sufficient to produce susceptibility. The animals of subsequent generations, which

lack one or more of these factors, are non-susceptible, and the ratio of the susceptible to the non-susceptible in the second filial generation should indicate the number of factors on which these characters are based. With the present material, Tumor J. w. A., however, the susceptible F_2 individuals are so rare that it would require much more abundant data than we have here presented to arrive at any conclusion concerning even the approximate number of factors necessary. It is probable that the number is rather large, for if susceptibility were based on from twelve to fourteen factors, as many positive animals would occur as we have obtained. Further experiments are at present being carried out with another tumor, which with the same stocks of mice promises to be more favorable in this respect. Since one of the characters studied (non-susceptibility) amounts to an ability to develop immunity to a given foreign cell, it will be of interest if the same principles are found to apply in the inheritance of susceptibility to infectious diseases.

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APPENDIX.

The behavior of eight pairs of Mendelian factors.

In the construction of a similar table to apply to factors determining tumor growth we should not be justified in using symbols indicating their dominant or recessive nature, but could represent actual conditions by symbols merely indicating unlikeness, as A^J and A^T , rather than A and a .

Wild mouse ACBDPWFS crossed with albino waltzer acbdpwfs.

F_1 generation ($AaCcBbDdPpWwFfSs$) all appear like wild mouse.

EXPECTED RATIO OF VARIOUS COLOR TYPES IN F_2 GENERATION.

6561....	A	C	B	D	P	W	F	S
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"	A	C	b	D	P	W	F	S
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729....	a	C	b	D	P	W	F	S
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"....	a	c	B	d	p	W	f	s
"....	a	c	B	d	p	w	F	s
"....	a	c	B	d	p	w	f	S
"....	a	c	b	D	P	w	f	s
"....	a	c	b	D	p	W	f	s
"....	a	c	b	D	p	w	F	s
"....	a	c	b	D	p	w	f	S
"....	a	b	c	d	P	W	f	s
"....	a	b	c	d	P	w	F	s
"....	a	b	c	d	P	w	f	S
"....	a	b	c	d	p	W	F	s
"....	a	b	c	d	p	W	f	S
"....	a	b	c	d	p	w	F	S

3....	A	b	c	d	p	w	f	s
"....	a	B	c	d	p	w	f	s
"....	a	b	C	d	p	w	f	s
"....	a	b	c	D	p	w	f	s
"....	a	b	c	d	P	w	f	s
"....	a	b	c	d	p	W	f	s
"....	a	b	c	d	p	w	F	s
"....	a	b	c	d	p	w	f	S

I....	a	b	c	d	p	w	f	s	albino (pink eyed dilute brown blz- spot waltz).
-------	---	---	---	---	---	---	---	---	--

I x I	I
8 x 3	24
28 x 9	252
56 x 27	1512
70 x 81	5670
56 x 243	13608
28 x 729	20412
8 x 2187	17496
I x 6561	6561
	<hr/>
	65536

CHARTS.

The following charts present the data obtained in the present series of experiments.

A carcinoma (J. w. A.) of the Japanese waltzing mouse was used in all instances.

Experiments A 1 to A 5 were carried out on mice of the *brown agouti* stock described elsewhere and their hybrids with the waltzing mice.

In Experiments B 1 to B 3 the *dilute brown* stock and their hybrids were employed.

The tumors are represented at approximately one-seventh of their actual diameter. In some instances the weight of the tumor is given at the time of death, which is otherwise indicated by the symbol †.

SUMMARY — EXPERIMENT A 1.

Eleven F₂ hybrids. — Two weeks after inoculation five show small nodules. Subsequently all negative.

Twelve F₃ hybrids (three died after six days). — Two and three weeks after inoculation three show nodules. Subsequently all retrogress, although one grows for five weeks and persists for over seven weeks.

Eleven (F₁ x Br. Ag.)¹ hybrids. — No appreciable growth of tumor.

Eleven Br. Ag. stock mice. — No growth.

Twelve (F₁ x J. w.)¹ hybrids (one died early). — Eleven grew large tumors.

Eleven F₁ hybrids. — All grew large tumors.

Eleven J. w. mice. — All positive.

SUMMARY — EXPERIMENT A 2.

Fifty F₂ hybrids. — Two weeks after inoculation twelve show nodules. Three weeks after inoculation ten show nodules. Four weeks after inoculation six show nodules. Five weeks after inoculation two show nodules. One of these retrogressed after twelve weeks, the other grew continuously.

Thirty-seven (F₁ x Br. Ag.)¹ hybrids. — No growth.

Six Br. Ag. stock mice. — No growth.

Twelve (F₁ x J. w.)¹ hybrids (seven died early). — Five grew large tumors.

Six F₁ hybrids. — One died five weeks later. Five positive.

Twelve J. w. mice. — Two weeks after inoculation all showed tumor nodules. Tumors grew until death, which occurred early.

Eight (F₁ x Br. Ag.)² hybrids. — Two weeks after inoculation two showed nodules. Three died on this date. Subsequently all negative.

SUMMARY — EXPERIMENT A 3.

Thirty-seven F_2 hybrids (seven died early). — Two weeks after inoculation eleven show nodules. Three weeks after inoculation five show nodules. Four weeks after inoculation three show nodules. Subsequently two of these retrogressed, but the other grew progressively.

Five F_3 hybrids. — Three weeks after inoculation one shows a minute nodule, but died on this date. All others negative.

Sixteen ($F_1 \times Br. Ag.$)¹ hybrids (one died early). — Two weeks after inoculation seven show nodules. Three weeks after inoculation three show nodules. Five weeks after inoculation two show nodules. Subsequently both tumors retrogressed.

Forty-one $Br. Ag.$ stock mice. — Two weeks after inoculation five show minute nodules. Three weeks after inoculation one shows minute nodule. Subsequently all negative.

Six ($F_1 \times J. w.$)¹ hybrids. — All positive.

Eleven F_1 hybrids. — All positive.

Three $J. w.$ mice (one died early). — Others positive.

Five ($F_1 \times Br. Ag.$)² hybrids. — Two weeks after inoculation one shows a nodule. Subsequently all negative.

Four ($F_1 \times F_2$)¹ hybrids. Three weeks after inoculation two show nodules. One subsequently retrogressed, the other continued to develop until death.

SUMMARY — EXPERIMENT A 4.

Eleven F_2 hybrids (three lost). — Two weeks after inoculation five show nodules. Three weeks after inoculation one shows nodules. Subsequently all negative.

Three F_3 hybrids (one died early). — Others negative.

Four F_1 hybrids. — All positive.

Six $J. w.$ mice. — All positive.

Three ($F_1 \times F_2$)¹ (one died early). — One positive.

SUMMARY — EXPERIMENT A 5.

Thirty-three F_2 hybrids. — Two weeks after inoculation seven show nodules. Three weeks after inoculation seven show nodules. Four weeks after inoculation three show nodules. Five weeks after inoculation two show nodules. Six weeks after inoculation one shows a nodule. Subsequently all negative.

Seven F_3 hybrids. — Two weeks after inoculation one shows a nodule. Subsequently all negative.

Seven ($F_1 \times Br. Ag.$)¹ hybrids. — No growth.

Twenty-six ($F_1 \times J. w.$)¹ hybrids. — All positive.

Ten F_1 hybrids. — All positive.

Six $J. w.$ mice. — All positive.

Nineteen ($F_1 \times Br. Ag.$)² hybrids. — No growth.

Three ($F_1 \times F_2$)¹ hybrids. — Two weeks after inoculation one shows a nodule. Subsequently all negative.

Five ($F_1 \times J. w.$)² hybrids. — Three weeks after inoculation two show nodules. One subsequently retrogressed. One positive.

SUMMARY — EXPERIMENT B 1.

Twenty-six F₂ hybrids (four died early). — Two weeks after inoculation six show nodules. Subsequently all negative.

Five t₃ hybrids (one died early). — Others negative.

Twenty-one d. Br. stock mice. — One showed a nodule for five weeks, then retrogressed. All negative.

Twenty-two F₁ hybrids (two died early). — One negative (not tested by re-inoculation). Nineteen positive.

Ten J. w. mice (two died early). — Others positive.

SUMMARY — EXPERIMENT B 2.

Twenty-three F₂ hybrids. — Eight show temporary growth from two to six weeks after inoculation. One positive — progressively growing tumor.

Nine (F₁ x d. Br.)¹ hybrids. — Two weeks after inoculation one shows a nodule. Subsequently all negative.

Ten d. Br. stock mice. — Three weeks after inoculation one shows a nodule. Subsequently all negative.

Six J. w. mice. — All positive.

SUMMARY — EXPERIMENT B 3.

Thirty-five F₂ hybrids (eight died early). — Eight show temporary growth from one to five weeks after inoculation. Subsequently all negative.

Eleven F₃ hybrids. — Two weeks after inoculation one shows a nodule. Subsequently all negative.

Eleven d. Br. stock mice. — Two weeks after inoculation one shows a nodule. Subsequently all negative.

Nineteen (F₁ x J. w.)¹ hybrids (four died early). — Others positive.

Five (F₁ x d. Br.)² hybrids. — Two weeks after inoculation two show nodules. Three weeks after inoculation one shows a nodule. Subsequently all negative.

Eight J. w. mice (one died early). — Others positive.

EXPERIMENT		A.I.	DAYS AFTER INOCULATION									DAYS AFTER INOCULATION						
No.	CLASS	AGE	14	21	28	35	42	49		No.	CLASS	AGE	14	21	28	35	42	49
4067	F ₂	2 Mo 15 d.	•	—	—	—	—	—		4110	(F ₁ × Jw) ¹	57 d.	•	•	•	•	4.654	
4068	"	"	•	—	—	—	—	—		4111	"	"	•	•	•	•	3.150	
4069	"	"	•	—	—	—	—	—		4112	"	"	•	•	•	•	1.512	
4090	"	"	•	—	—	—	—	—		4113	"	"	•	•	•	•	1.883	
4091	"	53 d.	—	—	—	—	—	—		4114	F ₁	4 Mo 28 d.	•	•	•	•	4.290	
4092	F ₃	2 Mo 1 d.	—	—	—	—	—	—		4115	"	"	•	•	•	•	7.152	
4093	"	"	—	—	—	—	—	—		4116	"	"	•	•	•	•	7.900	
4094	"	"	—	—	—	—	—	—		4117	"	"	•	•	•	•	6.250	
4095	"	19 d. + 5 d.	—	—	—	—	—	—		4118	"	5 Mo 9 d.	•	•	•	•	2.954	
4096	"	" + 5 d.	—	—	—	—	—	—		4119	Jw.	35 d.	•	•	•	•	•	•
4097	"	" + 5 d.	—	—	—	—	—	—		4120	"	"	•	†	—	—	—	—
4098	(F ₁ × B ₁)	57 d.	—	—	—	—	—	—		4121	"	"	•	•	•	•	•	•
4099	"	"	—	—	—	—	—	—		4122	"	35 d.	•	•	•	•	•	1050
4100	"	"	—	—	—	—	—	—		4123	"	"	•	•	†	370	—	—
4101	"	"	—	—	—	—	—	—		4124	F ₂	53 d.	—	—	—	—	—	—
4102	"	"	—	—	—	—	—	—		4125	"	"	•	—	—	—	—	—
4103	Br. Ag.	19 d.	—	—	†	—	—	—		4126	"	2 Mo 24 d.	—	—	—	—	—	—
4104	"	"	—	—	—	—	—	—		4127	"	"	—	—	—	—	—	—
4105	"	"	—	—	—	—	—	—		4128	"	"	—	—	—	—	—	—
4106	"	"	—	—	—	—	—	—		4129	"	"	—	—	—	—	—	—
4107	"	"	—	—	—	—	—	—		4130	F ₃	3 Mo 9 d.	•	†	65	—	—	—
4108	(F ₁ × Jw) ¹	57 d.	•	•	•	•	1.700	—		4131	"	19 d.	•	•	—	—	—	—
4109	"	"	•	•	†	1.540	—	—		4132	"	"	—	—	—	—	—	—

EXPERIMENT		A.2.	DAYS AFTER INOCULATION									DAYS AFTER INOCULATION						
No.	CLASS	AGE	14	21	28	35	42	49		No.	CLASS	AGE	14	21	28	35	42	49
4166	F ₂	47d.	—	—	—	—	—	—		4189	(F ₁ BrAg)	2Mo2d.	—	—	—	—	—	—
4167	"	"	—	—	—	—	—	—		4190	"	"	—	—	—	—	—	—
4168	"	"	—	—	—	—	—	—		4191	"	" 15d.	—	—	—	—	—	—
4169	"	"	—	—	—	—	—	—		4192	"	"	—	—	—	—	—	—
4170	"	3Mo3d.	—	—	—	—	—	—		4193	"	"	—	—	—	—	—	—
4171	"	"	—	—	—	—	—	—		4194	"	"	—	—	—	—	—	—
4172	"	" 5d.	—	—	—	—	—	†		4195	"	" 2d.	—	—	—	—	—	—
4173	"	2Mo15d.	—	—	—	—	—	—		4196	(F ₁ XJw)	33 d.	†	.03				
4174	"	"	•	•	—	—	—	—		4197	"	"	†	.066				
4175	"	"	—	—	—	—	—	—		4198	"	"	†	.7d 1.020				
4176	"	"	—	—	—	—	—	—		4199	"	"	†	.053				
4177	"	3Mo28d.	†							4200	F ₁	8Mo28d.	•	•	•	•	1.725	
4178	"	"	—	—	—	—	—	—		4201	"	"	†	2d.				
4179	"	" 18d.	—	—	—	—	—	—		4202	"	4Mo29d.	•	†	307			
4180	"	2Mo12d.	—	—	—	—	—	—		4203	"	"	•	•	•	•	3.190	
4181	"	" 5d.	—	—	—	—	—	—		4204	"	"	•	•	•	•	1.047	
4182	"	"	•	•	•	—	—	—		4205	"	"	•	•	•	•	3.558	
4183	(F ₁ BrAg)	" 4d.	—	—	—	—	—	—		4206	F ₂	25 d.	•	•	•	—	—	—
4184	"	"	—	—	—	—	—	—		4207	"	"	—	—	—	—	—	—
4185	"	"	—	—	—	—	—	—		4208	"	"	—	—	—	—	—	—
4186	"	"	—	—	—	—	—	—		4209	"	"	—	—	—	—	—	—
4187	"	"	—	—	—	—	—	—		4210	"	"	—	—	—	—	—	—
4188	"	"	—	—	—	—	—	—		4211	"	"	•	•	•	•	•	•

EXPERIMENT		A.2. Cont.		DAYS AFTER INOCULATION								DAYS AFTER INOCULATION					
No.	CLASS	AGE	14	21	28	35	42	49	No.	CLASS	AGE	14	21	28	35	42	49
4212	F ₂	25 d	1.00						4235	(F ₂ JW) ¹	33 d	●	●	●	●	3.50	
4213	"	"	●	●	●	●	●	●	4236	"	"	↑					
			●56d	●63d	●77d	●84d	●98d										
4214	"	"	—	—	—	—	—	—	4237	"	33 d	●	●	●	●	6.50	
4215	"	"	—	—	—	—	—	—	4238	"	"	↑	0.13				
4216	"	"	●	—	—	—	—	—	4239	(F ₂ Ba ₂) ²	28 d	↑					
4217	"	"	—	—	—	—	—	—	4240	"	"	—	↑				
4218	"	33 d	●	●	●	—	—	—	4241	"	"	●	●	—	—	—	—
4219	"	"	—	—	—	—	—	—	4242	"	"	↑					
4220	"	"	●	—	—	—	—	—	4243	"	"	—	—	—	—	—	—
4221	"	2Ma23d	—	—	—	—	—	—	4244	"	"	—	—	—	—	—	—
4222	"	"	—	—	—	—	—	—	4245	"	"	—	—	—	—	—	—
4223	(F ₂ Ba ₂) ¹	59 d	—	—	—	—	—	—	4246	"	"	↑	0.10				
4224	"	"	—	—	—	—	—	—	4247	F ₂	26 d	↑	5d				
4225	"	"	—	—	—	—	—	—	4248	"	"	●	●	●	—	—	—
4226	"	2Ma2d	—	—	—	—	—	—	4249	"	"	↑	5d				
4227	"	"	—	—	—	—	—	—	4250	"	"	↑	5d				
4228	"	"	—	↑					4251	"	38 d	●	●	—	—	—	—
4229	"	"	—	—	—	—	—	—	4252	"	"	—	—	—	—	—	—
4230	"	"	—	—	—	—	—	—	4253	"	"	—	—	—	—	—	—
4231	"	"	—	—	—	—	—	—	4254	"	"	—	—	—	—	—	—
4232	"	"	—	—	—	—	—	—	4255	"	25 d	●	●	●	●	—	—
4233	"	"	—	—	—	—	—	—	4256	"	36 d	—	—	—	—	—	—
4234	"	"	—	—	—	—	—	—	4257	"	"	—	—	—	—	—	—

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EXPERIMENT		A.2. Cont.		DAYS AFTER INOCULATION					
No.	CLASS	AGE	14	21	28	35	42	49	
4258	Fr	36 d.	—	—	—	—	—	—	
4259	"	"	●	—	—	—	—	—	
4260	"	"	—	—	—	—	—	—	
4261	"	"	●	●	—	—	—	—	
4262	"	"	—	—	—	—	—	—	
4263	(FrBrAg)	3Mo30 d.	—	—	—	—	—	—	
4264	"	47 d.	—	—	—	—	—	—	
4265	"	"	—	—	—	—	—	—	
4266	"	"	—	—	—	—	—	—	
4267	"	"	—	—	—	—	—	—	
4268	"	"	—	—	—	—	—	—	
4269	"	33 d.	—	—	—	—	—	—	
4270	"	"	—	—	—	—	—	—	
4271	"	"	—	—	—	—	—	—	
4272	"	"	—	—	—	—	—	—	
4273	"	"	—	—	—	—	—	—	
4274	"	"	—	—	—	—	—	—	
4275	(FrJw)	2Mo5 d.	●	●	●	●	2.320		
4276	"	"	●	●	●	●	.811		
4277	"	3Mo30 d.	●	●	●	●	1.350		
4278	"	2Mo2d †							
4279	BrAg.	31 d.	—	—	—	—	—	—	
4280	"	"	—	—	—	—	—	—	

EXPERIMENT		A.2. Cont.		DAYS AFTER INOCULATION					
No.	CLASS	AGE	14	21	28	35	42	49	
4281	BrAg.	31 d.	—	—	—	—	—	—	
4282	"	"	—	—	—	—	—	—	
4283	"	"	—	—	—	—	—	—	
4284	"	"	—	—	—	—	—	—	
4285	Jw.	49 d.	—	●	●	●	●	† .352	
4286	"	"	●	† .086					
4287	"	"	●	† .068					
4288	"	"	●	† .077					
4289	"	"	●	† .230					
4290	"	"	●	† .035					
4291	"	"	●	† .057					
4292	"	"	●	† .016					
4293	"	"	●	† .079					
4294	"	"	●	† .180					
4295	"	"	●	† .033					
4296	"	"	●	† .092					

EXPERIMENT A.3			DAYS AFTER INOCULATION										DAYS AFTER INOCULATION						
No.	CLASS	AGE	14	21	28	35	49	56		No.	CLASS	AGE	14	21	28	35	49	56	
4386	F ₂	24 d.	—	—	—	—	—	—		4409	F ₂	38 d.	—	—	—	—	—	—	
4387	"	"	—	—	—	—	—	—		4410	"	"	—	—	—	—	—	—	
4388	"	"	—	†	—	—	—	—		4411	"	"	—	—	—	—	—	—	
4389	"	"	—	—	—	—	—	—		4412	"	27 d. †	7d.	—	—	—	—	—	
4390	"	"	—	—	—	—	—	—		4413	"	"	†	6d.	—	—	—	—	
4391	F ₂	32 d.	—	—	—	—	—	—		4414	"	32 d. †	6d.	—	—	—	—	—	
4392	"	20 d.	—	—	—	—	—	—		4415	"	23 d. †	9d.	—	—	—	—	—	
4393	"	26 d.	—	—	—	—	—	—		4416	"	20 d. †	7d.	—	—	—	—	—	
4394	"	"	—	—	—	—	—	—		4417	"	"	†	9d.	—	—	—	—	
4395	"	20 d. †	7d.	—	—	—	—	—		4418	(F ₁ B ₂ A ₂)	"	—	—	—	—	—	—	
4396	"	31 d.	—	—	—	—	—	—		4419	"	"	—	—	—	—	—	—	
4397	"	"	—	—	—	—	—	—		4420	"	"	—	—	—	—	—	—	
4398	"	"	—	—	—	—	—	—		4421	"	"	—	—	—	—	—	—	
4399	"	"	—	—	—	—	—	—		4422	"	"	—	—	—	—	—	—	
4400	"	"	—	—	—	—	—	—		4423	(F ₁ B ₂ A ₂)	33 d.	—	—	—	—	—	—	
4401	"	"	—	—	—	—	—	—		4424	"	"	—	—	—	—	—	—	
4402	"	"	—	—	—	—	—	—		4425	"	24 d.	—	—	—	—	—	—	
4403	"	"	—	—	—	—	—	—		4426	"	"	—	—	—	—	—	—	
4404	"	"	—	—	—	—	—	—		4427	"	"	—	—	—	—	—	—	
4405	"	38 d.	—	—	—	—	—	—		4428	"	"	—	—	—	—	—	—	
4406	"	"	—	—	—	—	—	—		4429	"	"	—	—	—	—	—	—	
4407	"	"	—	—	—	—	—	—		4430	"	"	—	—	—	—	—	—	
4408	"	"	—	—	—	—	—	—		4431	"	"	—	—	—	—	—	—	

EXPERIMENT		A.3. Cont.		DAYS AFTER INOCULATION										DAYS AFTER INOCULATION					
No.	CLASS	AGE	14	21	28	35	49	56		No.	CLASS	AGE	14	21	28	35	49	56	
4432	(FrBrAg)	24 d	•	•	•	•	—	—		4455	BrAg	25 d	—	—	—	—	—	—	
4433	"	59 d	—	—	—	—	—	—		4456	"	"	—	—	—	—	—	—	
4434	"	"	—	—	—	—	—	—		4457	"	"	—	—	—	—	—	—	
4435	"	36 d + 8 d	—	—	—	—	—	—		4458	"	"	—	—	—	—	—	—	
4436	"	42 d	—	—	—	—	—	—		4459	"	"	—	—	—	—	—	—	
4437	"	"	—	—	—	—	—	—		4460	"	42 d	—	—	—	—	—	—	
4438	"	25 d	—	—	—	—	—	—		4461	"	"	—	—	—	—	—	—	
4439	BrAg	23 d	—	—	—	—	—	—		4462	"	"	—	—	—	—	—	—	
4440	"	"	—	—	—	—	—	—		4463	"	"	—	—	—	—	—	—	
4441	"	"	—	—	—	—	—	—		4464	"	"	—	—	—	—	—	—	
4442	"	"	—	—	—	—	—	—		4465	"	"	—	—	—	—	—	—	
4443	"	"	—	—	—	—	—	—		4466	"	"	•	•	—	—	—	—	
4444	"	"	—	—	—	—	—	—		4467	"	"	—	—	—	—	—	—	
4445	"	"	—	—	—	—	—	—		4468	"	"	—	—	—	—	—	†	
4446	"	"	—	—	—	—	—	—		4469	"	" + 8 d	—	—	—	—	—	—	
4447	"	"	—	—	—	—	—	—		4470	"	"	—	—	—	—	—	—	
4448	"	"	—	—	—	—	—	—		4471	"	"	—	—	—	—	—	—	
4449	"	"	—	—	—	—	—	—		4472	"	"	—	—	—	—	—	—	
4450	"	25 d	—	—	—	—	—	—		4473	"	"	—	—	—	—	—	—	
4451	"	"	—	—	—	—	—	—		4474	"	"	—	—	—	—	—	† 48 d	
4452	"	"	—	—	—	—	—	—		4475	"	"	—	—	—	—	—	—	
4453	"	"	—	—	—	—	—	—		4476	"	"	—	—	—	—	—	—	
4454	"	"	—	—	—	—	—	—		4477	"	"	—	—	—	—	—	—	

EXPERIMENT A.5.		DAYS AFTER INOCULATION									DAYS AFTER INOCULATION						
No.	CLASS	AGE	14	21	28	35	42	49	No.	CLASS	AGE	14	21	28	35	42	49
4712	F ₂	34 d.	•	•	—	—	—	—	4735	F ₂	2Mo5d.	—	—	—	—	—	†
4713	"	"	•	•	—	—	—	—	4736	(F ₂ Jw) [†]	41 d.	—	—	—	—	—	—
4714	"	"	•	•	—	—	—	—	4737	"	"	—	—	—	—	—	—
4715	"	"	•	•	—	—	—	—	4738	"	"	—	—	—	—	—	†
4716	"	49 d.	—	—	—	—	—	—	4739	"	26 d.	•	•	•	•	•	† .607
4717	"	"	—	—	—	—	†	—	4740	"	"	—	—	—	—	—	†
4718	"	"	—	—	—	—	†	—	4741	(F ₂ BzAg) [†]	45 d.	—	—	—	—	—	—
4719	"	"	•	•	•	•	•	—	4742	"	"	—	—	—	—	—	—
4720	"	"	—	—	—	—	—	—	4743	"	"	—	—	—	—	—	—
4721	"	"	—	—	—	—	—	—	4744	"	"	—	—	—	—	—	—
4722	"	46 d.	—	—	—	—	—	—	4745	"	"	—	—	—	—	—	†
4723	"	"	—	—	—	—	—	—	4746	(F ₂ F ₂) [†]	41 d.	—	—	—	—	—	†
4724	"	"	—	—	—	—	—	—	4747	"	"	—	—	—	—	—	†
4725	"	44 d.	—	—	—	—	—	—	4748	"	"	—	—	—	—	—	†
4726	"	"	•	•	•	†	—	—	4749	(F ₂ Jw) [†]	36 d.	•	—	•	—	† Eaten	—
4727	"	"	—	—	—	—	—	—	4750	"	"	•	•	•	•	•	.073
4728	"	"	•	•	†	—	—	—	4751	"	"	•	•	•	•	•	.250
4729	F ₂	58 d.	—	—	—	—	—	—	4752	"	"	—	•	•	•	•	.745
4730	"	"	—	—	—	—	—	—	4753	"	"	—	•	•	•	•	—
4731	"	"	—	—	—	—	—	—	4754	"	45 d.	•	•	•	•	•	.2200
4732	"	"	•	—	—	—	†	—	4755	"	"	•	•	•	•	•	1.055
4733	"	2Mo5d.	—	—	—	†	—	—	4756	"	"	•	•	•	•	•	1.260
4734	"	"	—	—	—	—	—	—	4757	"	"	•	•	•	•	•	.763

EXPERIMENT			A.S.	Cont.	DAYS AFTER INOCULATION							No.	CLASS	AGE	DAYS AFTER INOCULATION						
No.	CLASS	AGE	14	21	28	35	42	49		14	21				28	35	42	49			
4758	F ₁	45 d.	•	•	•	•	•	735		4781	F ₂	60 d.	—	—	—	—	—	—			
4759	"	"	•	•	•	•	•	Partly eaten		4782	"	"	—	—	†	—	—	—			
4760	"	"	•	•	•	•	•	2915		4783	"	"	—	—	—	—	—	—			
4761	"	"	•	•	•	•	•	1342		4784	"	41 d.	—	—	—	—	—	†			
4762	"	"	•	•	•	•	•	1325		4785	"	"	—	—	—	—	—	—			
4763	(F ₁ B ₁ A ₁)	56 d.	—	—	—	†	—	—		4786	"	48 d.	—	—	—	—	—	—			
4764	"	"	—	—	—	—	—	†		4787	"	"	—	—	—	—	—	—			
4765	"	"	—	—	—	—	—	—		4788	"	"	—	—	—	—	—	—			
4766	"	"	—	—	—	—	—	—		4789	"	"	—	—	—	—	—	—			
4767	"	"	—	—	—	—	—	—		4790	"	"	—	—	—	—	†	—			
4768	"	25 d.	—	—	—	—	—	—		4791	"	"	—	—	—	—	—	—			
4769	"	"	—	—	—	—	—	†		4792	(F ₁ B ₁ A ₁)	57 d.	—	—	—	—	—	—			
4770	J.w.	58 d.	•	•	•	•	•	2300		4793	"	"	—	—	—	—	†	—			
4771	"	"	•	•	•	•	•	2215		4794	"	"	—	—	—	†	—	—			
4772	"	"	•	•	•	•	•	—		4795	"	30 d.	—	—	—	—	—	—			
4773	"	"	•	•	•	•	•	3467		4796	"	"	—	—	—	—	—	—			
4774	"	"	•	•	•	•	•	—		4797	"	"	—	—	—	—	—	—			
4775	"	"	•	•	•	•	•	—		4798	"	"	—	—	—	—	—	—			
4776	F ₂	46 d.	—	—	—	—	—	—		4799	"	"	—	—	—	—	†	—			
4777	"	60 d.	—	—	—	†	—	—		4800	"	2Mo3d.	—	—	—	—	—	—			
4778	"	"	—	—	—	†	—	—		4801	"	38 d.	—	—	—	†	—	—			
4779	"	"	—	—	—	†	—	—		4802	"	"	—	—	—	—	—	—			
4780	"	"	—	—	—	†	—	—		4803	"	"	—	—	—	—	—	—			

No.	CLASS	AGE	DAYS AFTER INOCULATION				
			14	21	28	35	42
4883	F ₁	45 d.	•	•	●	●	.850
4824	"	"	•	•	●	●	.358
4825	"	"	•	•	●	●	.480
4826	"	"	•	•	●	●	.575
4827	"	"	—	•	●	●	1.015

EXPERIMENT		B.I.	DAYS AFTER INOCULATION									DAYS AFTER INOCULATION						
No.	CLASS	AGE	14	21	28	35	42	49		No.	CLASS	AGE	14	21	28	35	42	49
4297	F ₃	41 d. †	3d.							4320	F ₃	41 d.	●	—	—	—	—	—
4298	"	"	—	—	—	—	—	—		4321	"	31 d.	—	—	—	—	—	—
4299	"	"	—	—	—	—	—	—		4322	"	"	—	—	—	—	—	—
4300	"	"	—	—	—	—	—	—		4323	"	"	—	—	—	—	—	—
4301	"	"	—	—	—	—	—	—		4324	"	"	●	—	—	—	—	—
4302	F ₃	5 Mo 12 d.	●	—	—	—	†			4325	"	"	—	—	—	—	—	—
4303	"	"	●	—	—	—	—	—		4326	"	"	—	—	—	—	—	—
4304	"	" 5 d.	●	—	—	—	—	—		4327	d.Br.	41 d.	—	—	—	—	—	—
4305	"	" 8 d.	—	—	—	—	—	—		4328	"	"	—	—	—	—	—	—
4306	"	2 Mo 10 d.	—	—	—	—	—	—		4329	"	"	—	—	—	—	—	—
4307	"	"	—	—	—	—	—	—		4330	"	"	—	—	—	—	—	—
4308	"	"	—	—	—	—	—	—		4331	"	"	—	—	—	—	—	—
4309	"	"	—	—	—	—	—	—		4332	"	"	—	—	—	—	—	—
4310	"	"	—	—	—	—	—	—		4333	"	31 d.	—	—	—	—	—	—
4311	"	"	—	—	—	—	—	—		4334	"	"	—	—	—	—	—	—
4312	"	23 d. †	3d.							4335	"	"	—	—	—	—	—	—
4313	"	" †	3d.							4336	"	"	—	—	—	—	—	—
4314	"	" †	3d.							4337	"	"	—	—	—	—	—	—
4315	"	" †	3d.							4338	"	"	—	●	●	●	—	—
4316	"	2 Mo 8 d.	—	—	—	—	—	—		4339	"	"	—	—	—	—	—	—
4317	"	41 d.	—	—	—	—	—	—		4340	"	"	—	—	—	—	—	—
4318	"	"	—	—	—	—	—	—		4341	"	"	—	—	—	—	—	—
4319	"	"	—	—	—	—	—	—		4342	"	"	—	—	—	—	—	—

(70d)
1.775

EXPERIMENT B2									DAYS AFTER INOCULATION									DAYS AFTER INOCULATION								
No.	CLASS	AGE	14	21	35	42	49	56	No.	CLASS	AGE	14	21	35	42	49	56									
4531	F ₈	28 d.	●	●	—	—	—	—	4555	(F ₈ d.Br) [†]	46 d.	—	—	—	—	—	—									
4532	"	41 d.	●	—	—	—	—	—	4556	"	"	●	—	—	—	—	—									
4533	"	"	●	—	—	—	—	—	4557	"	"	—	—	—	—	—	—									
4534	"	"	—	—	—	—	—	—	4558	"	"	—	—	—	—	—	—									
4535	"	37 d.	●	—	—	—	—	—	4559	"	"	—	—	—	—	—	—									
4536	"	28 d.	●	●	●	●	—	—	4560	"	"	—	—	—	—	—	—									
4537	"	"	—	—	—	—	—	—	4561	"	"	—	—	—	—	—	—									
4538	"	41 d.	●	—	—	—	—	—	4562	"	"	—	—	—	—	—	—									
4539	"	"	—	—	—	—	—	—	4563	d.Br	5Mo12d.	—	—	—	—	—	—									
4540	"	"	—	—	—	—	—	—	4564	"	"	●	●	—	—	—	—									
4541	"	"	—	—	—	—	—	—	4565	"	"	—	—	—	—	—	—									
4542	"	44 d.	●	—	—	—	—	—	4566	"	12Mo12d.	—	—	—	—	—	—									
4543	"	21 d. †	—	—	—	—	—	—	4567	"	56 d.	—	—	—	—	—	—									
4544	"	" † 9 d.	—	—	—	—	—	—	4568	"	2Mo7d.	—	—	—	—	—	—									
4545	"	44 d.	—	—	—	—	—	—	4569	"	"	—	—	—	—	—	—									
4546	"	"	—	—	—	—	—	—	4570	"	"	—	—	—	—	—	—									
4547	"	"	—	—	—	—	—	—	4571	"	"	—	—	—	—	—	—									
4548	"	"	—	—	—	—	—	—	4572	"	45 d.	—	—	—	—	—	—									
4549	"	37 d.	—	—	—	—	—	—	4573	J.w.	41 d.	—	—	—	—	—	—									
4550	"	"	—	—	—	—	—	—	4574	"	"	●	●	—	—	—	—									
4551	"	"	—	—	—	—	—	—	4575	"	"	●	●	—	—	—	—									
4552	"	44 d.	—	—	—	—	—	—	4576	"	"	●	●	—	—	—	—									
4553	"	"	—	—	—	—	—	—	4577	"	"	—	—	—	—	—	—									
4554	(F ₈ d.Br) [†]	46 d.	—	—	—	—	—	—	4578	"	"	—	—	—	—	—	—									

EXPERIMENT B.3.			DAYS AFTER INOCULATION										DAYS AFTER INOCULATION						
No.	CLASS	AGE	14	21	28	35	42	49		No.	CLASS	AGE	14	21	28	35	42	49	
4623	F ₃	26 d.	†							4646	F ₃	25 d.	—	—	—	—	—	—	
4624	"	"	† Eaten							4647	"	"	—	—	—	—	—	—	
4625	"	"	—	—	—	—	—	—		4648	"	"	—	—	—	—	—	—	
4626	"	"	† 9 d							4649	"	"	—	—	—	—	—	—	
4627	"	42 d.	•	•	•	•	†	—		4650	"	"	—	—	—	—	—	—	
4628	"	"	•	•	—	—	—	—		4651	"	"	—	—	—	—	—	†	
4629	"	37 d.	—	—	—	—	—	—		4652	"	58 d.	—	—	—	—	—	—	
4630	"	"	—	—	—	•	† •	—		4653	"	"	—	—	—	—	—	—	
4631	"	58 d.	† 9 d							4654	"	"	—	†					
4632	"	"	—	—	—	—	—	—		4655	"	"	† 7 d						
4633	"	34 d.	—	—	—	—	—	—		4656	"	"	—	—	—	—	—	—	
4634	"	"	—	—	—	—	†	—		4657	"	"	—	—	—	—	—	—	
4635	"	"	—	—	—	—	—	—		4658	F ₃	53 d.	—	—	—	—	—	—	
4636	"	"	—	† •						4659	"	"	—	—	—	—	—	—	
4637	"	"	† 4 d							4660	"	"	—	—	—	—	—	—	
4638	"	"	—	—	—	—	—	—		4661	"	"	—	—	—	—	—	—	
4639	"	"	—	•	—	—	—	—		4662	"	2 Mo 6 d.	—	—	—	—	—	—	
4640	"	37 d.	—	—	—	—	—	—		4663	"	"	—	—	—	—	—	—	
4641	"	"	—	—	—	—	—	—		4664	"	"	—	—	—	—	—	—	
4642	"	"	—	—	—	—	—	—		4665	"	"	—	—	—	•	—	—	
4643	"	"	—	—	—	—	—	—		4666	"	"	—	—	—	—	—	—	
4644	"	52 d.	—	—	—	—	—	—		4667	"	27 d.	—	—	—	—	—	—	
4645	"	"	—	—	—	—	—	—		4668	"	"	—	—	—	—	—	—	

453

DAYS AFTER INOCULATION

No.	CLASS	AGE	DAYS AFTER INOCULATION					
			14	21	28	35	42	49
4669	(F+Jw)	52 d.	-	-	-	-	-	-
4670	"	"	-	-	-	-	-	-
4671	"	28 d.	-	-	-	-	-	-
4672	"	"	-	-	-	-	-	-
4673	"	"	-	-	-	-	-	-
4674	d.Br.	2 Mo. 6 d.	-	-	-	-	-	-
4675	"	"	-	-	-	-	-	-
4676	"	"	-	-	-	-	-	-
4677	"	"	-	-	-	-	-	-
4678	"	"	-	-	-	-	-	-
4679	"	"	-	-	-	-	-	-
4680	"	"	-	-	-	-	-	-
4681	"	"	-	-	-	-	-	-
4682	"	"	-	-	-	-	-	-
4683	"	"	-	-	-	-	-	-
4684	"	"	-	-	-	-	-	-
4685	(F+Jw)	38 d.	-	-	-	-	-	-
4686	"	"	-	-	-	-	-	-
4687	"	"	-	-	-	-	-	-
4688	"	"	-	-	-	-	-	-
4689	"	48 d.	-	-	-	-	-	-
4690	"	"	-	-	-	-	-	-
4691	"	"	-	-	-	-	-	-

No.	CLASS	AGE	14	21	28	35	42	49
4692	(F+Jw)	34 d.	-	-	-	-	-	-
4693	"	"	-	-	-	-	-	-
4694	"	9 d.	-	-	-	-	-	-
4695	"	"	-	-	-	-	-	-
4696	"	"	-	-	-	-	-	-
4697	"	31 d.	-	-	-	-	-	-
4698	"	"	-	-	-	-	-	-
4699	"	"	-	-	-	-	-	-
4700	"	2 Mo. 1 d.	-	-	-	-	-	-
4701	"	"	-	-	-	-	-	-
4702	"	"	-	-	-	-	-	-
4703	"	"	-	-	-	-	-	-
4704	Jw.	50 d.	-	-	-	-	-	-
4705	"	"	-	-	-	-	-	-
4706	"	"	-	-	-	-	-	-
4707	"	"	-	-	-	-	-	-
4708	"	9 d.	-	-	-	-	-	-
4709	"	"	-	-	-	-	-	-
4710	"	"	-	-	-	-	-	-
4711	"	"	-	-	-	-	-	-

OLD AGE IN RELATION TO CELL-OVERGROWTH
AND CANCER.*

E. W. GOODPASTURE AND G. B. WISLOCKI.

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The condition of multiple tumor formation in many organs and tissues of old dogs, which we shall describe, has an important significance we believe in relation to the age incidence of cancer in man and lower animals. It illustrates strikingly the tendency of cells of senescent organs to take on new growth which may result in a malignant tumor. Although our series of animals is yet small, it is fairly complete in that we have found tumor formation in nearly every glandular organ, and in other tissues of the body. This fact has induced us to describe the condition at this time even though incompletely, postponing a more thorough account of the histological changes preceding tumor formation, which we can follow with some clearness in the thyroid and perhaps other glands, until further studies are made.

That old age prepares a way for the development of cancer is one of the most familiar facts in our fragmentary knowledge of tumor genesis. The various writers who have proposed hypotheses to explain the origin of malignant new growths have emphasized or almost ignored the age incidence of cancer, according to the adequacy of their individual views to give a rational interpretation of it. (Bashford (*vide infra*) gives a valuable critical analysis of various theories of cancer origin in the light of experimental results.) Thus Cohnheim's familiar theory accorded it little attention, while Ribbert's somewhat more flexible hypothesis permitted it more prominence; and Freund has so adjusted Thiersch's conception of a state of equilibrium existing between connective tissue and epithelium that his modification of the theory emphasizes the more rapid aging of connective tissue as an explanation in cancer formation of its loss of

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normal restraint upon the inherent proliferative powers of epithelium. Bashford,¹ perhaps more than any other, has emphasized senescence as a causative factor in the origin of cancer and has extended the law of age incidence to include the malignant tumors of many mammals and lower vertebrates. His investigations and those of many other workers upon tumors in lower animals have served to increase our knowledge greatly within recent years. The recognition of such new growths as quite analogous to those of man has placed our conception of tumors in general upon a broader biological foundation and enlarged the field for further advance. The difficulties in following cellular changes leading to tumor formation, and in observing the transition from orderly, perhaps highly differentiated, functioning cells into malignant neoplasms, in human tissues are obvious. But now such problems are open to attack with relative ease in laboratory animals, for in the dog tumors occur in such numbers, variety, and seemingly with such regularity in old individuals that transition stages may be easily encountered which would be quite accidentally found in human beings and perhaps never subject to controlling observation. Partially, perhaps as a consequence of these difficulties in accurately following consecutive senescent changes, the importance of old age as a causative factor of cancer origin has not received the prominence it may deserve. In this connection Adami² has suggested that "possibly the tendency to development of glandular cancer in later life bears some relationship to the reversion and degeneration of gland cells at this period. As the tissues become exhausted the more highly differentiated cells tend to become structurally simpler, revert, that is to say, to a simpler type, and with this simplification of structure accompanying atrophy there may be a greater liability for those cells to assume proliferative powers, etc." Whatever may be the ultimate incentive to tumor formation and malignant growth, there can be little doubt that senescence predisposes cells to take on this activity and that this predisposition, in the dog at least we hope to show, is general in perhaps all glandular and

connective tissues. An explanation of the coincident generalization of such changes in dogs possibly may be found in the relatively brief individual existence of these animals and in the persistence of active reproductive organs well into old age.

Although reports of malignant and benign tumors of dogs are numerous,³ we have not found that this very general and seemingly constant tendency to new growths in old individuals has been described, certainly it has not received the attention we feel it merits.

Among autopsies performed in the Hunterian Laboratory during the past winter our attention was attracted by finding multiple tumors in very old dogs arising in many tissues, especially those of glandular structure. The apparent uniqueness of the condition led us to examine with great interest the fifteen old animals which have been at our disposal, and in each instance a similar picture has presented itself. In our series nearly every organ of the body has been the seat of multiple tumor formation. Each animal has shown involvement of several organs, varying, however, in different individuals. The liver with one exception contained tumors, the adrenal gland in all but a single instance, the other organs in different proportions. The character of the alterations in the same organ has been uniform in all individuals; and we are convinced that the process is fundamentally a series of cellular changes dependent upon functional involution of an organ or tissue during senescence, the picture varying with the nature of the cells which are the seat of this change.

In only one case was it possible for us to learn even an approximate age of the animal. It had been in the possession of its owner for fourteen years, and judging from appearances few of those in which we have found tumors were younger. We have been directed in our diagnosis of senility by familiar evidences such as the loss of teeth, presence of cataract, and the appearance of decrepitude. Most of our animals, however, have shown only the absence of front teeth and wasting of others as a conspicuous sign. In

no animal with extensive wasting and loss of teeth have we failed to find tumors in more than one organ. We may say also that we have never failed to find multiple tumors in every dog which we have classified as old before autopsy, and in hundreds of normal younger animals we have never observed a similar condition. All the old dogs were mongrels varying greatly in size and general features. We shall describe briefly the characteristic alterations met with in the individual organs and tissues.

Liver. — The liver is slightly smaller than normal, its color a light brown, or yellowish brown, sometimes with a greenish tint. Its surface is smooth, but made irregular by the projection of round or oval, externally somewhat flattened tumors which have the general appearance of liver parenchyma. These nodules vary in size from two millimeters to three centimeters in diameter and in number from three to twenty. The surface tumors complete a more or less spherical contour in the liver tissue beneath. They are not confined to the surface, but may be found on cut section irregularly scattered throughout the interior. More minutely they have the appearance in general of liver parenchyma, although there is no regular lobulation and the columns of cells are larger than in the normal tissue. The cells are usually more yellow and opaque than those in the adjoining parenchyma, but they may be, especially when small and in the interior, quite reddish, meaty, and translucent. Blood capillaries and sinuses between the cell strands are prominent. There is no connective tissue capsule, but the structure of such a tumor and the appearance of its cells suffice to define sharply its outline.

Microscopically these tumors are seen to be composed of liver parenchyma, with no very sharp contrast to the liver cells about them except sometimes a thin zone of compressed and atrophic cells surrounding their periphery. There is no regular lobulation, the bile ducts are present here and there, accompanied by their blood vessels. The bile capillaries are atrophic and surrounded or completely replaced by small

round cells. No bile formation is evident in the tumor cells. Individually the cells are full of fine doubly refractive fat droplets. Irregular patches of large neutral fat globules, both intra and extra-cellular, are present in the tumor, but more numerous in the surrounding cells. In addition to fat droplets the cells contain many small vacuoles which do not stain as fat, and hyaline particles staining with eosin.

Gall bladder and bile ducts. — The interior of the gall bladder is rough from the projection of small papillomatous and cystic ingrowths of its mucous membrane. The papillomatous folds may be three or four millimeters long. They are lined by high columnar epithelium which secretes abundant mucus. Small cysts two to four millimeters in diameter filled with clear yellowish fluid are numerous. In some cases numerous small cystic dilatations of bile ducts are found scattered through the liver, especially near the surface. Degeneration of smaller bile ducts and capillaries is evident as described above.

Adrenals. — The adrenal glands are larger than normal and their surface is irregularly nodular from smooth, more or less spherical protuberances which vary in size from one millimeter to two centimeters in diameter. These tumors are multiple and occur in both glands. On section they are seen to occupy and project from the cortex and are composed of cortical tissue. Their composition is more compact and their color more yellow and opaque than the surrounding cortex. There is no definite structure to be seen in them on section, the cut surface appearing quite homogeneous. The medulla is usually compressed, sometimes invaded by a small tumor nodule of cortical origin. These tumors are round or oval structures sharply defined by the compact arrangement, the absence of normal architecture, and the greater fat content of their large cells. There is no connective tissue capsule surrounding them.

Microscopically the larger tumors are found to arise from the cells of the fascicular zone. This zone may be hypertrophic, its cells being larger than normal, and containing

more fat, or there may be extensive areas of cortical atrophy. The glomerular zone in the dog is more distinct in its structure than in man. It is composed of columnar epithelium arranged in more or less regular concentric groups or short columns surrounded by definite connective tissue strands. Its cells contain much less fat than do those of the fascicular or reticular zone. In our animals this zone is nearly always the seat of focal proliferative changes, with invasions of the capsule forming small protrusions onto the surface.

The cells of the fascicular zone tumors are larger than normal and are more densely packed with doubly refractive fat globules.

Degenerative changes are to be seen in many cells, both in the tumors and in the surrounding cortex. These consist in the presence of abnormally large fat globules and clear vacuoles within the cytoplasm, and of shrunken, often pyknotic, nuclei. Occasionally a small area of cortex has undergone necrosis forming a cyst-like space surrounded by cortical cells presenting a picture with a superficial resemblance to an acinus. In one tumor of fascicular zone origin there are definite acinus-like structures containing a granular material apparently secreted by the surrounding cells. Small hemorrhages are not infrequently found, especially near the medulla, and in the neighborhood of these are large phagocytes containing granules of blood pigment. The lymphatics about larger veins are often distended with cells filled with fat globules and vacuoles. One cortical tumor has the structure of an alveolar sarcoma, an architecture which may be met with in certain so-called Grawitz tumors.

In one case (Dog 8) there is a malignant tumor of the right adrenal arising from the medulla. The medulla is greatly enlarged and completely occupied by the tumor which invades the cortex slightly on all sides, growing through it anteriorly into the lumbar vein, protruding along its lumen even into the vena cava. The tumor tissue is quite distinct from the cortex in its appearance. It is firm, gray, and translucent except near its central portions, which contain yellow opaque areas. No normal medulla is seen. The

arrangement of the tumor cells is compact with strands of connective tissue tracing through, forming a sort of alveolar architecture. The cell nuclei are small, round or oval, and where they are densely packed together they assume an oblong outline. The cytoplasm is indistinct. In areas where the cells are not compact the nuclei are round and the cytoplasm is reticular and branching. In the central portion of that part of the growth which has invaded the lumbar vein there are large areas of hyaline degeneration with spots of calcification. Here and there are small blood sinuses lined by tumor cells. The character and arrangement of the tumor cells resembles in general that of the normal medulla.

In the inferior median margin of the right lobe of the liver in the same case are four firm gray translucent nodules measuring one-half centimeter in diameter. The liver tissue between them has atrophied. No other nodules were found on section of the organ. These nodules are composed of cells of the same appearance as those of the adrenal medulla tumor and are undoubtedly its metastases.

Spleen. — The spleen is about normal in size. In the majority of cases smooth, tense, spherical, deep purple tumors protrude from the surface. They vary in number from one to six in each spleen and in size from one-half to three centimeters in diameter. They are a little softer in consistency than the normal splenic tissue and on section are seen to be composed of large, irregular, gray and translucent compact cellular masses, separated and surrounded by deep purple depressed areas. The gray masses are densely packed groups of lymphocytes with large proliferating centers. Large blood sinuses are frequently present in and about these groups. In one case at least the spleen pulp in the neighborhood of such a lymphoma shows bone marrow activity. There are numerous megalokaryocytes, myelocytes, and nucleated red blood cells in the neighborhood of the tumors.

In general the lymphatic tissue of these animals is atrophic, though in two cases glands from the base of the mesentery show hyperplasia of the lymph follicles.

Prostate. — This gland in each instance is greatly hypertrophied, usually being two or three times its normal size. The surface is irregularly nodular, some of the nodules having the appearance of small cysts and contain a clear viscous fluid. The enlargement is evidently due to a hyperplasia of the epithelium and dilation of the alveoli. The epithelial lining of the alveoli forms extensive papillary ingrowth. The cells are for the most part high columnar in character, though some spaces are lined by a cuboidal or even flat type, or even by all three varieties. One enlarged prostate presents a different picture. Lining epithelium has almost completely desquamated, and many dilated tubules show it falling away en masse leaving a single layer of small cuboidal cells. These latter cells are budding and filling the organ with immature ducts.

Ovary. — Multiple cysts are present in each ovary, the largest not exceeding one centimeter in diameter. These contain clear fluid. Microscopically they possess a thin capsule of connective tissue and are lined by a single layer of flat cells. In three ovaries corpora lutea are present and several Graafian follicles show marked hyperplastic changes. The change begins apparently with the death and disintegration of an ovum. The follicular epithelium increases in abundance and the cells tend to become columnar. The lining infolds, the folds being followed by connective tissue stroma. With continued proliferation and extension a tumor is formed consisting in cross-section of a network of papillary processes supported by connective tissue stroma. These tumors may measure three centimeters in diameter. They are multiple. The epithelial cells become high columnar in type closely arranged with nuclei at the bases. Apparently normal follicles are present in each of these three animals.

Thyroid. — In five animals multiple tumors were present in the thyroid gland; in three of these both glands contained tumors. The right thyroid of Dog 11 is almost completely replaced by a gray translucent spherical nodule measuring

2.5 centimeters in diameter. The number of tumors in each gland varies from one to six, and in size up to 2.5 centimeters in diameter. They are definitely circumscribed, round or oval, gray or yellowish gray compact nodules. The larger ones show a sort of myxomatous degeneration in the centers accompanied by hemorrhage, or blood sinuses.

The larger tumors may or may not be surrounded by a definite connective tissue capsule from which trabeculae extend into the tumor separating it roughly into large lobules. The tumor cells present a variety of appearances. The most common architecture is that of a tubular or columnar arrangement of large, more or less columnar cells with abundant granular cytoplasm. There is no colloid in these areas, and mitotic figures are very numerous. Another appearance which may be present in the same tumor is that of a fetal adenoma. Small cells with very little cytoplasm are arranged in irregular masses and columns with an extremely delicate sometimes myxomatous connective tissue stroma. The masses and columns of cells may be very loose in structure and separated by irregular spaces. These cells tend to arrange themselves into small acini, variable in size, containing colloid. Blood vessels are rare. Blood sinuses lined by tumor cells are seen. The process of formation of these tumors may be followed to some extent. There is first a disintegration of colloid in an area consisting of one or more acini. The colloid shrinks, stains blue with hematoxylin and becomes broken up into many small round bodies. The acini shrink, and the lining cells then begin to grow larger and more columnar, there being relatively much more cytoplasm than normal. The cells then begin to proliferate, filling up the acinar spaces until the whole group of acini is a small tumor. These tumors apparently are enlarged partly by the accretion of similar neighboring areas of proliferation, partly by proliferation of their own cells.

Stomach.—In five of the animals multiple polyps were present in the pylorus. They appear as cauliflower growths of the mucosa from one-half to two centimeters in diameter,

the larger ones extending two centimeters above the mucous membrane. There are from two to ten present in each stomach. They occur only in the pylorus. Microscopically they are composed of finger-like processes of mucous membrane surrounding a connective tissue core. The cells are columnar and secrete mucus. There is no downward growth of cells.

Testes. — In two animals multiple tumors of the testes were present. These are of two types, namely, those with the architecture of the tubular portion, and those composed of cells resembling the interstitial cells of Leydig. The largest tumor of the latter variety measures three centimeters in diameter, being practically spherical. On opening the capsule of the testis this mass bulged out as if under tension. On cut surface it resembled very much a mass of brain substance, soft, yellowish, gray, and pink. It is definitely circumscribed, although there is no capsule surrounding it. The tumors of the tubular type are firmer and measure up to two centimeters in diameter. They have a smooth cut surface, compact grayish yellow tissue. They were the more numerous in each animal. In both cases there were multiple tumors in each testis.

Microscopically the interstitial cell tumors are very cellular growths with very little connective tissue stroma, many capillaries, and blood spaces. The individual cells are like the interstitial cells, though larger as a rule. The nuclei are round or oval, do not stain very deeply and contain a definite nucleolus. There is an abundant cytoplasm filled with lipid granules, and sometimes containing yellow particles. There is great variation in the size of these cells, and mitoses are fairly numerous. Another structure is quite numerous and seems to be derived from nuclei by a process of vacuolation. These are irregularly oval or round bodies, vesicular with a thin hematoxylin staining membrane and colorless contents. Nuclei are seen which contain a large vacuole stretching the nuclear membrane and it would seem that the vesicular bodies are derived from these.

In some places the cells are more compactly arranged and are small. They may be radially situated about a blood space, and have a spindle structure.

The tumors resembling the tubular portion of the testis are composed of compact, irregularly arranged, immature tubules about which there is a connective tissue stroma. The tubules may be solid columns of cells or may be lined by several layers of cells with large, round, or oval nuclei lightly staining, with distinct nucleolus and abundant branching cytoplasm which tends to send out wavy thread-like processes to the center. Mitotic figures are occasionally seen.

In the remaining testicular tissue no spermatogenesis is found, though there is great proliferation of the lining cells in places.

Pancreas. — Macroscopically this gland is of normal size, its lobulation distinct and there is no evidence of increase in the connective tissue stroma. The parenchyma is a little firmer than normal. Scattered irregularly over the surface are numerous opaque white areas of parenchyma about the size of a lobule. These are composed of several acini in which the epithelial cells are large, with nuclei near the basement membrane; their prominent cytoplasm stains a bright pink with eosin, and is very granular and vacuolated. One such area in Dog 4 is definitely circumscribed by a loose interacinar connective tissue. The acini and cells are much larger than normal.

These characteristic spots are most probably due to occlusion of small ducts by proliferating duct epithelium, inasmuch as one finds many places in which small ducts are proliferating, forming new buds and structures quite similar to islands of Langerhans.

The islands appear normal.

Thymus. — Hammar⁴ in his studies upon the thymus describes its involutionary changes in the dog, and mentions the hyperplasia of its epithelial elements which accompanies its metamorphosis. Early in life there begin to appear in the

gland duct-like spaces lined completely or partially by ciliated, cuboidal or columnar epithelium and containing an albuminous material. Coincidentally with their increase in number Hassall's corpuscles disappear. Hammar believes the ciliated cells are direct modifications of reticular cells. Recently Marine⁵ has advocated the view that they are remnants of the embryonal thymic buds. That the former view is probably correct, we believe, a tumor of the reticular cells, which we shall describe below, will indicate.

As age progresses and the lymphoid cells and Hassall's corpuscles decrease in number, the duct like spaces become more numerous and increase in size until in old age we find the thymus composed of a number of cysts about one-fourth centimeter in diameter containing a clear yellow viscous fluid. The wall of these cysts is lined by cuboidal cells which may or may not be ciliated. In between them small patches of lymphoid and reticular cells with perhaps a Hassall's body still may persist. The cystic thymus is the most advanced stage of involution we have found. At a somewhat earlier period the reticular cells are apparently very active. The gland at this time consists largely of duct-like spaces with irregularly infolded lining partially of ciliated columnar cells and partially of reticular cells. Buds of reticular cells may be seen penetrating the surrounding fatty tissue. Duct-like spaces form in or follow these buds apparently by a process of degeneration of the central cells. Well defined patches of lymphoid tissue are present, and still a few Hassall's bodies.

At such a stage in Dog 11 we find a tumor in the thymus, discrete, oval in outline, measuring 2 x 1 centimeters, and surrounded by a connective tissue capsule. The tumor consists of very compactly arranged cells containing oval nuclei with one or more minute chromatin granules, and a finely reticular cytoplasm. Microscopically, the tumor possesses numerous spaces ranging in size from small degenerated areas involving a few cells to relatively large dilated duct-like structures lined partially or completely by cuboidal, ciliated epithelium. Blood vessels are not prominent,

though hemorrhage has occurred in some of the smaller spaces. There are no Hassall's corpuscles. About the periphery of the tumor are the dilated duct-like spaces, and patches of lymphoid cells of the remaining thymus.

It seems certain that this tumor is a new growth of the reticular cells, and it affords an excellent opportunity to study the formation of the duct-like spaces, and the origin of the ciliated cells, as the process is in all probability the same in the tumor as in the involuting gland.

The first change in the formation of a space takes place in a single tumor cell. This cell enlarges; its cytoplasm becomes greatly increased in amount and filled with pink staining granules; sometimes it contains also a small globule of colloid-like material. Gradually the nucleus becomes more and more isolated from the surrounding cells. It lies in a mass of granular cytoplasm which connects with the adjoining cells by cytoplasmic processes separated by vacuoles. These adjoining cells now begin to differentiate along the margin nearest the central cell. This inner margin becomes more regular; the cells assume a concentric arrangement, and tend towards a cuboidal shape. One or two of them develop more cytoplasm in the part next to the growing space. This cytoplasm fills with eosin-staining granules; then ciliated processes appear. The central cell undergoes degeneration; its cytoplasm becomes full of hyaline globules and vacuoles; its nucleus disintegrates by karyorrhexis. Other marginal cells are thrown off into the space after undergoing a similar process. The ciliated cells evidently secrete an albuminous material which fills the spaces, and it would seem as if the cilia are really threads of this secretion, for in certain places they are very long and are gradually lost in a mass of pink staining granular material; and those spaces which contain no granular secretion have no ciliated cells. Other spaces contain a more homogeneous material of a colloid appearance, and colloid-like globules are found in the cytoplasm of some cells. These colloid spaces are not lined by ciliated cells.

It is evident then that reticular cells may under certain

conditions be transformed directly into the ciliated form, which has to all appearances a secretory activity.

Salivary glands. — In Dog 13 there was a large cyst in and about the left parotid gland. Growing into this cyst from the duct, and completely plugging it, was a large papillomatous growth composed of finger-like processes covered by duct epithelium and containing a core of connective tissue and blood vessels. In another case small papillomatous growths were found in the ducts of the submaxillary gland.

Sebaceous glands. — There was a tumor in Dog 9 measuring 3 x 2 centimeters in the skin of the external ear. The tumor consisted in an overgrowth of sebaceous gland epithelium, forming nests of cells and small cysts. The tumor was evidently a very slow growing type.

Mammary gland. — In only two cases have we encountered breast tumors; both were the mixed tumor variety, composed of an overgrowth of glandular epithelium with irregular tubule formation, and of cartilage. The larger tumor was as large as a hen's egg and increased noticeably in size while the animal was nursing pups.

Hypophysis. — The hypophysis of Dog 8 is greatly enlarged. A tumor in the anterior portion measures $1\frac{1}{2}$ x 1 centimeters. It is firm and on section gray and translucent. The growth encroaches upon the overlying brain, the sella remaining normal.

Microscopically the tumor appears to be a great hyperplasia of the pars intermedia. In large areas acinar structure is predominant. The acini have the size and appearance of those normally found in the pars intermedia and contain a colloid material. The cleft is filled with colloid. In other portions acini are absent and the growth is composed of closely arranged, indistinct, and irregular columns of cells. No acidophil or basophil cells are found in it. A compressed zone of anterior lobe with very numerous acidophil cells is present at its anterior periphery. The tumor is not surrounded by a connective tissue capsule.

Parathyroids.—No definite hyperplasia has been noted in these glands. Certain degenerative changes perhaps are worthy of mention. One gland in Dog 4 has undergone cystic degeneration. The cyst is lined by a single layer of low cuboidal epithelium; the interior consists of granular débris. In Dog 1 the glands are somewhat larger than normal and there are areas in one of them composed of from ten to twenty columns in which the cells, though arranged as usual about small blood vessels, are very large. The cytoplasm appears as a single large vacuole surrounded by a thin membrane. These cells contain normal-looking nuclei.

Adipose tissue.—Many of these old dogs, especially females, are obese. It has been of great interest to find in four animals multiple subcutaneous lipomata; and one of these dogs was an emaciated male. There are three or four such tumors present in each, varying in size up to that of a hen's egg. They are smooth, spherical or oval tumors, rather firm and quite colorless, standing in contrast to the yellowish normal fat. They are composed of closely arranged fat cells with very little connective tissue stroma. Some of the fatty contents is doubly refractive. The situation of these tumors is quite variable. In one animal a small lipoma was situated anteriorly at the base of the right ventricle.

Connective tissue.—In Dog 9 a fibroma one and one-half centimeters in diameter was found near the cardiac end of the stomach in the lesser curvature. Another smaller one was present on the posterior wall of the bladder.

Blood vessels.—In Dog 9 a hemangioma typical in structure was present beneath the skin in the lateral wall of the abdomen. It measured two centimeters in diameter. It is interesting to note that the intima of the aorta and larger arteries shows no athero-sclerosis in any of these animals.

CHART.

Dog No.	Sex.	Organs.														
		Liver.	Gall Bladder.	Stomach.	Pancreas.	Parotid.	Submaxillary.	Spleen.	Adrenals.	Testes.	Prostate.	Ovary.	Breast.	Thyroid.	1 hymus.	Hypophysis.
1 . .	F.	T	T	T	T	..	T
2 . .	F.	T	T	T	T	T	T	..	T
3 . .	F.	T	T	T	T	T	T
4 . .	F.	T	T	T	T	T
5 . .	F.	..	T	T	T	T	T
6 . .	F.	T	T	T	T	T
7 . .	F.	T	T	T
8 . .	M.	T	T	T	T	..	T	T	..
9 . .	M.	T	T	T	T	..	T	T	..
10 . .	M.	T	T	T	..	T
11 . .	M.	T	T	T	T	..	T	T	T	..
12 . .	M.	T	..	T	T	..	T
13 . .	M.	T	T	T	..	T	T	T	T	T	T	T
14 . .	M.	T	T	T	T	T
15 . .	M.	T	T	T	T	T	T
15	14	8	5	1	1	2	14	14	2	8	5	2	5	1	1
															4	1

The above chart shows that tumors are present in a number of organs in each animal. Their uniform and coincident occurrence, the fact that they are usually multiple in each organ, and a certain similarity in their mode of formation and growth make us feel that they are an expression of a common cause, the picture varying with the character of the tissue in which they occur. There are indications that the process begins as a regressive change, that is to say, certain cells first cease to function and later these areas take on new growth. This may be followed best in the thyroid. Here there is first a cessation of function in multiple foci consisting of a few acini. This is indicated by disintegration of colloid and shrinking of the acini. The epithelial cells become larger

by an increase of cytoplasm which is more granular apparently than normal. In some of these acini, before their lumina have completely disappeared and while atypically staining granules of colloid still persist, we find evidence already of beginning proliferation in the increase in number of cells at points along the epithelial linings. Later the former acinus becomes a solid column of epithelium. The atrophic area is thus converted into a small tumor which increases in size both by proliferation of its own cells and by coalescing with other similar areas.

Though we may not follow with equal clearness the progress of these changes in all the organs, we do find in most instances quite comparable phenomena. In the ovary a degeneration of ova, in the testicle absence of spermatogenesis, in the liver atrophy of bile capillaries, and an absence of bile formation in the tumors, atrophy of the adrenal cortex, and of lymphoid tissue, accompany focal proliferative changes in these tissues. Both degenerative and proliferative changes are usually multiple in each organ and we do not often find proliferation without evidences of degeneration.

Thus we are led to the interpretation that proliferation leading to tumor formation occurs in these cases in the cells of functionally involuting tissues; and since the changes occur simultaneously in so many parts of the body it is only natural to assume a common cause for their origin. We can imagine no other than senescence. Assuming, however, an equal age for these animals we do not find that each organ in the various cases presents corresponding changes, nor are the same organs affected in each instance. This is to be explained we think by a variable absolute longevity of organs and tissues of an individual. As the individual longevity of different species varies tremendously, and as the functional life of certain organs of the same individual is relatively short, so the period of functional existence of each organ and tissue of different individuals of the same species probably has a variable limit, which may be shortened or lengthened perhaps by circumstances of the individual life. Consequently an organ of one individual may earlier show

evidences of disintegration than the same organ of another, and with the disintegration a tendency to proliferate.

In a general way the process seems to us to be a mode of disorganization of the individual, a coincident involution of constituent groups of units, units which even at the expense of the individual, sustained by their harmonious function, may under certain conditions strive to perpetuate their own life as more or less independent organisms, when the natural period of their ability to function expires.

All the new growths we have described, except the adrenal medulla tumor which metastasized to the liver, were benign in the sense that they did not extensively invade surrounding structures, nor metastasize. Whether they would eventually exhibit these malignant characters, and in what proportion, our series is too small to permit us to say. Surgical pathology has taught us, however, that such apparently benign overgrowths as senile parenchymatous hypertrophy and intra-cystic papilloma of the breast, both of which have a maximum incidence during functional involution of the gland, are dangerous precancerous lesions. Certainly the histological evidences of rapid growth such as some of the above-described dog tumors present (very numerous mitotic figures, variations in size, shape and staining reactions of cells) indicate the possibility of rapid extension.

We have purposely made no attempt to classify the various tumor forms, preferring to look upon them simply as manifestations of a progressive process rather than as more or less distinct morphological units.

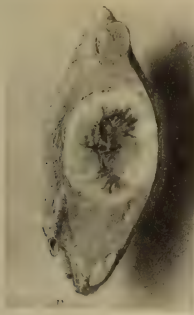
[We wish to thank Miss Helen Lorraine and Dr. Harry Clough for their assistance in preparing the illustrations.]

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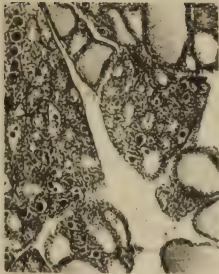
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EXPLANATION OF PLATE XVIII.

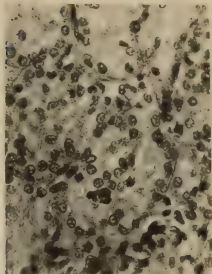
- THYROID:** FIG. 1. — Tumors of thyroid.
 FIG. 2. — Area of degeneration leading to tumor formation.
 FIG. 3. — Thyroid tumor showing active mitosis.
- ADRENAL:** FIG. 4. — Tumor of adrenal medulla invading lumbar vein and inferior vena cava.
- THYMUS:** FIG. 5. — Thymus of old dog showing irregular tubule formations lined partially by ciliated epithelium, and rapidly becoming cystic. Small area of lymphoid cells, containing smaller ducts and very few Hassall's corpuscles.
 FIG. 6. — Duct-like space in thymus tumor lined partially by ciliated epithelium.
- TESTES:** FIG. 7. — Larger tumor is composed of cells resembling the interstitial cells of Leydig, smaller gray tumor of tubular portion.
 FIG. 8. — Section of interstitial cell tumor showing lipoid granules in the cells.
 FIG. 9. — Section through tumor arising from tubular cells of testis.
- HYPOPHYSIS:** FIG. 10. — Section through tumor showing abundant acini containing a colloid material.
- SPLEEN:** FIG. 11. — Lymphoma of spleen.
- STOMACH:** FIG. 12. — Papillomatous overgrowths of pyloric mucosa.



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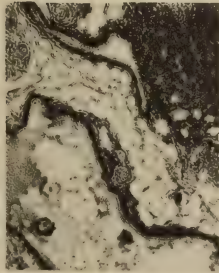
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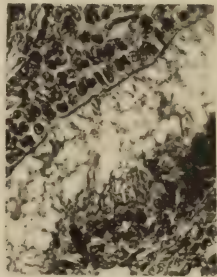
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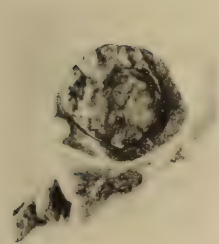
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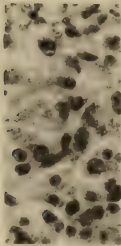
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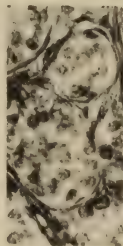
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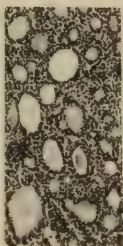
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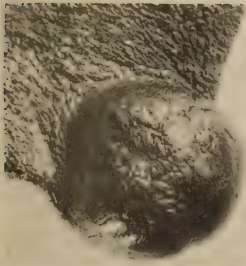
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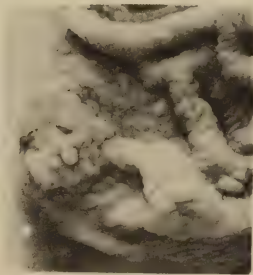
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THE ACCUMULATION OF ANISOTROPIC FATS IN
INTERSTITIAL CELLS OF THE KIDNEY.*

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Although the subject of artificial feeding of cholesterin has been extensively investigated of late, we have seen no record of the effect of such experiments upon the kidney. While engaged in experiments of administering cholesterin to rabbits we noted certain definite changes in the interstitial tissue of the renal medulla.

We are familiar with the tissue changes which have been described in the experimental feeding of lipoids. The establishment of cholesterin, as the causative factor of the changes produced in feeding experiments, was proved by Stuckey and Wesselkin. Anitschkow has demonstrated the development of fatty plaques in the aorta of cholesterin-fed rabbits. Rothschild showed the relation of the Kupffer cells of the liver and Sternberg the function of the adrenal in cholesterin metabolism. Chalatow noted the laying down of cholesterin-esters in the liver, while Aschoff and Landau have called attention to the importance of endothelial tissue in dealing with cholesterin materials. We found in our own work, during a study of the nature of the lesion produced by cholesterin in the aorta of rabbits, organic response similar to that reported by other authors, and, in addition, noted that the kidneys of some of our animals showed a change which was unfamiliar to us.

The animals were daily fed by a stomach tube with cholesterin, some animals receiving the cholesterin in olive oil, while a sodium oleate cholesterin emulsion was fed to others. Five of the animals were given a daily dose of cholesterin varying from .28 to .56 gram. Two of these

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rabbits, R. 2 and R. 3, showed no macroscopical evidence of change in the aorta while the kidneys were affected. Animals R. 4, R. 6, and R. 9 had developed fatty plaques in their aortas as well as changes in the kidneys. Rabbit 9 exhibited the most marked production of fatty intimal change in aorta and also the most intense studding over of the medullary portion of its kidneys by small white areas. Another rabbit, R. 12, receiving seventy-five grams of cholesterin in nineteen days showed no change in its aorta or kidneys in the gross. Although the kidneys of R. 12 were negative in the gross there was microscopic evidence of change simulating the cellular reaction noted in the kidneys of our other animals.

Animal.	Days Treated.	Daily Dose of Cholesterin.	Total Cholesterin.	Fatty Streaks in Aorta.	Change in Medulla of Kidney.
<i>Cholesterin Olive Oil.</i>					
R. 2 . . .	57	0.28	8.12	None.	Present.
R. 3 . . .	61	0.28	15.96	"	"
R. 4 . . .	86	0.28-0.56	31.92	Well developed.	Well developed.
R. 6 . . .	108	0.28-0.56	44.24	" "	" "
<i>Sodium Oleate Cholesterin.</i>					
R. 9 . . .	115	0.56	61.6	Very marked.	Very marked.
R. 12 . .	19	3.94	75	None.	Present.

Macroscopically the reaction was localized in the pyramids of the renal medulla. When the organ was sectioned from pole to pole yellowish white streaks marked the pyramids. These were arranged radially below the intermediate zone. They bulged upon the cut surface and their direction was that of the collecting tubules. On cross section of the organ these areas were seen as milk-white dots scattered through the tissue.

Microscopically these white areas were confined to the interstitial tissue of the pyramids. The earliest alterations were noted in the kidneys of R. 12. There was a beginning proliferation of the lining cells of the small capillaries. As

each successive animal received more cholesterol over a longer period of time the proliferation of the interstitial cells became more pronounced. The most advanced lesions were observed in rabbit R. 9. Paraffin sections showed the production of islands of cells growing between the tubules and compressing them. The cells in these areas were large and irregular, their shape depending upon their close arrangement. Some were round, while again others were elongated with flattened sides. Their protoplasm was swollen and made up of a delicate network, between the meshes of which there were small spaces. At times quite large vacuoles were present in the protoplasm. The nuclei as a rule were round, quite small, and deeply stained. However, in some cells the nuclei were larger and somewhat vesicular. In most instances the cells were surrounded by well-defined membranes, although at times several cells were fused together and the individual cell membranes could not be made out. Between the large cells red blood corpuscles were frequently seen. In fact, the structure had the appearance of small capillary channels with a proliferation of the lining endothelium. The renal tubules in the vicinity of these areas were easily recognized and were bounded by intact basement membranes (Mallory). Some of the tubules were dilated and lined by flattened cells. The epithelial cells of these tubules commonly presented a granular hydropic protoplasm.

In the earlier stages of proliferation of the interstitial cells the Sudan and hematoxylin staining brought out features which were not observed in the paraffin preparations. Throughout the interstitial tissue of the medulla, the lining endothelial cells of the small capillaries contained small Sudan globules. In one animal (R. 12) the endothelial cells of the glomerular tufts contained Sudan material with a fair amount of the same substance in the lining cells of the small interstitial vessels. When the proliferation of the lining cells of the capillaries was advanced, so that islands of cells were formed, the reaction resembled the accumulation of cells within the intimal fatty plaques of the aorta. The cells were large and swollen, and their bodies filled with Sudan globules

giving them a beaded appearance (foam cell). Where the cell membranes were not distinct, a large fatty mass was formed through which the deeply stained nuclei were seen. Occasionally an overloaded cell liberated some of its fatty content into the surrounding tissue. The fat within the foam cells was anisotropic.

In the lining cells of some of the tubules, close to the proliferation in the interstitial tissue of the pyramids, small globular Sudan material was observed. The desquamated cells lying within the tubules frequently contained Sudan stained globules. However, in comparison with the amount of fat locally stored by the endothelial islands, the cells of the tubules were remarkably free.

Aside from the above interstitial reaction there was another reaction which occurred in three of the kidneys, R. 4, R. 6, and R. 9. This reaction consisted of a proliferation of cells in the cortex. These cells were about the size of the lining cells of the convoluted tubules. They had a homogeneous cloudy protoplasm and centrally placed round nuclei. The direct relation of these cells to the tubules could be established by the use of Mallory's stain. It appeared as though the lining cells, by proliferation, had distorted and dilated the tubule. In one area tubular proliferation had occurred about a glomerulus. In the Sudan and hematoxylin sections a number of the above described cells contained granular Sudan material.

A proliferation of cells within the interstitial tissue of the pyramids of the kidney is quite a unique condition. From the location of the islands of cells scattered through the interstitial tissue of the pyramids, particularly in its upper half, attention is drawn to the possible relation of this proliferation with the lining cells of the medullary capillaries descending from the arcuate vessels. In our observation of the earliest response the lining cells of the small arterioles in this region showed some proliferation and already contained fat. In our subsequent animals where sufficient time for the proliferation of cells of the arterioles had elapsed, islands of cells were formed. The animals, R. 4, R. 6, and

R. 9, which presented the most marked lesion in the kidney, also showed well developed fatty plaques in the aorta. Again, in animals R. 2, R. 3, and R. 12, where there were no fatty plaques in the aorta, there were other evidences of a similar reaction. The response was also present in the Kupffer cells of the liver, the lining cells of the small arterioles of the heart, the arterial sinuses of the spleen and the arteriæ rectæ of the kidney. Thus it would seem that the endothelium of the more delicate vessels was first affected. When a considerable time is afforded to the experiments the endothelial tissues respond to the continued hypercholesterinemia by the development of areas which can be recognized in the gross, just as the appearance of the fatty plaques in the aorta. We are of the opinion that the reaction observed in the interstitial tissue of the pyramids of the kidneys was a response of the endothelial cells of the arteriæ rectæ and their capillaries. Furthermore, this proliferation has occurred as a part of a general response of endothelial tissues to the excess of cholesterin compounds in the blood.

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TISSUE REACTIONS IN EXPERIMENTAL HYPER-
CHOLESTERINEMIA.*

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Experiments performed by feeding cholesterin and foods rich in cholesterin to rabbits are attended by definite changes within the organs of these animals. This work was first undertaken by Ignatowski, who thought the changes in his animals were due to a severe disturbance of their protein metabolism. However, Stuckey found that he could produce changes in the aorta of rabbits by feeding them with egg yolk and brain substance, while he was unable to obtain results in rabbits fed upon pure neutral fats of animal or vegetable origin. Chalataw also investigated the effect of egg yolk and brain when used as food for rabbits and found an accumulation of doubly refractile substances within the liver cells with some evidence of cirrhosis. With substances devoid of cholesterin he found the organs of the animals without change. This author later observed that the feeding of pure cholesterin produced results similar to his previous experiments with egg yolk and brain substance. The results which were obtained with egg yolk led Wesselkin to use pure lecithin in an effort to produce these changes. However, his experiments were attended with negative results. On the other hand, Anitschkow employed pure cholesterin as a food for rabbits and found that fatty plaques were developed in the aorta of his animals.

In experiments similar to the above Wacker and Hueck determined that the cholesterin and cholesterin-ester content of the blood serum was increased. Rothschild, Waltmann and Biach, and Sternberg have obtained similar results.

In an effort to produce fatty plaques in the aorta like those observed by the Russian school we fed rabbits through a

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stomach tube with cholesterin in solution. Some of the rabbits received the cholesterin in olive oil, while others were given a sodium oleate cholesterin emulsion (Klotz). The daily dose of cholesterin varied from .28 to .56 gram. Of ten rabbits treated in this manner five showed the presence of fatty plaques in the aorta, while the organs of the remaining animals presented tissue changes of varying degrees.

The individual organs of animals, fed artificially with cholesterin, are not equally concerned in handling the cholesterin. This material calls forth a reaction dependent in its severity upon the amount administered and the length of time it is given. The changes noted consisted of an alteration in parenchymatous structures along with a response of endothelial tissue. Further, there was a marked accumulation of cholesterin and its compounds in some of the organs.

The organ which appears to be more concerned with cholesterin metabolism than any other is the adrenal. Shortly after the development of a hypercholesterinemia, the cells of the gland showed the beginning of an accumulation of cholesterin. This continued until after a period of one hundred and sixty-six days, as in one of our rabbits the organ reached a very large size, being two centimeters in diameter. The cortex of the gland became very much widened while the medulla was little affected. In all of the animals the adrenals presented various degrees of enlargement depending upon the amount of cholesterin they had received. The cells of the zona fasciculata and adjacent cells of the zona reticularis were particularly affected. The enlargement of the cortex depended upon the increase in size of its individual cells. They gradually increased in size until they became large and watery looking. Their protoplasm was either formed of a delicate reticulum or was fairly granular. The character of the protoplasm was a very interesting feature in the sections as the cells which had a reticulated protoplasm contained large numbers of cholesterol crystals and small and large-sized globules. Some of the cells showed fusion of the globules and were occupied by a single large vacuole. Again the cells which had a

granular protoplasm contained only small amounts of cholesterol crystals. It appeared as though the cells with the reticulated protoplasm were more intimately concerned with the storing up of the cholesterol materials. In sections stained with Sudan the cells were found to be loaded with an orange-colored fat. The Nile blue reagent showed most of this fat blue, although red-tinged globules were also present. The cells of the zona glomerulosa were filled with fat in the more advanced cases. The important part which the adrenal undertakes in endeavoring to store the cholesterol compounds circulating in the blood was demonstrated by the amount of anisotropic lipoid in the adrenals of these animals. Ordinarily the gland contains relatively little anisotropic lipoid when compared to the enormous amount of doubly refractile substances seen in the adrenals of a treated animal. Landau has pointed out that in rabbits fed with cholesterol, the adrenal receives the cholesterol in a combined form from the blood and endeavors by a process of storage to relieve the body of the excessive amount of cholesterol in the blood. Sternberg noted that there was no hyperplasia of the cortical cells but rather an overloading of the individual cells with cholesterol materials. From what we have observed in our sections we are inclined to believe that the adrenal is used as a depot for cholesterol compounds where it can be stored to relieve the blood of an excessive load.

Another organ which bears a very close relation to cholesterol metabolism is the liver. Not only are the liver cells themselves intimately concerned with the storage of the cholesterol compounds, but the lining cells of the sinusoids also contain lipoids and very often show proliferation. In the liver of a rabbit treated with seventy-five grams of cholesterol in nineteen days, the Kupffer cells stood out very prominently. They contained a considerable amount of lipoid material and were often loaded with fat globules while the liver cells were free. The part played by the Kupffer cells in the liver of this rabbit was so prominent that the fatty material had the appearance of being arranged in rows

which were limited to the sinusoids. The liver cells first contained fat in that part of the cell bordering the sinusoid and then later became filled with fat. It looked as though the liver cell obtained the fat from the Kupffer cell after the latter had taken it up from the blood. This relation of Kupffer cell to liver cell was first noted by Anitschkow and later studied by Rothschild. The fatty substances within the liver cells were mostly found within the inner half of the lobule. In this region the liver cells stored large quantities of fat so that the liver cells became vacuolated and often presented evidences of degeneration. The liver of this animal contained many more doubly refractile bodies than the adrenal. In animals which had been fed large amounts of cholesterin over a long period of time, the fatty accumulation within the cells gradually occupied more of the lobule. With polarized light the cells showed the doubly refractile globules in abundance, while many crystals and true plates of cholesterin were also noted. With maintenance of a hypercholesterinemia over a long period of time the liver accumulates an enormous amount of cholesterin compounds in an endeavor to furthermore relieve the body of the excess of cholesterin material in the blood. Rothschild has found that the liver secreted an excess of cholesterin in the bile when a sufficient grade of hypercholesterinemia was established. A morphological and chemical increase of cholesterin in the liver has been noted by Anitschkow and Chalatow and also by Weltmann and Biach.

The function of storing cholesterin is also undertaken by the spleen. This organ does not present the uniform evidence of a response of its tissue as in the case of the adrenal and the liver. Although this is broadly true, we found the organ responded very rapidly when the cholesterin feeding was forced. A rabbit fed with seventy-five grams of cholesterin in nineteen days showed a most unusual picture in its spleen. The organ was twice its normal size, quite firm and of a lighter color than usual. Paraffin sections of the organ showed the sinuses filled with large cells. Many of them

were large and foam-like, while others were definitely phagocytic and contained many acid and basic tinged granules along with a brown pigment which contained iron (Nishimura). Eosinophiles were frequently observed. This reaction was a part of the proliferative reaction of the lining cells of the sinuses which in some was so advanced that they were almost closed. This change was suggestive of the alteration seen in Gaucher's spleen. In Sudan sections the organ was loaded with orange stained fatty substances within the large cells filling the sinuses. A small amount of this fat was anisotropic. The reaction which occurred in the spleen of this animal was greater than that noted in any of the other rabbits. However, in some of the animals treated a longer time the spleen contained more anisotropic lipoid. The alterations noted in all of the spleens were limited to the arterial sinuses. Only occasionally were the arteries of the spleen affected and then the change consisted in a fatty degeneration of the media. The intima was not affected.

A structure which was actively concerned in the using up of the cholesterin was the corpus luteum. Two of our rabbits were pregnant during the feeding experiments. One of these animals received 76.72 grams of cholesterin in olive oil in one hundred and sixty-six days, while the other was fed 61.6 grams of cholesterin in a sodium oleate emulsion over a period of one hundred and fifteen days. Their ovaries were almost entirely occupied by corpora luteal tissue, only a small rim of ovarian structure containing Graafian follicles remained. As in the adrenal two types of cells were noted. The great majority of cells were large and had a granular reticulated protoplasm; other cells were seen with a delicately vacuolated meshwork in their protoplasm. In the latter many cholesterin clefts were observed, while the cells of the former type rarely contained them. The cells which contained cholesterin clefts were of the foam type and appeared to be more actively engaged in caring for cholesterin materials. They had no relationship with the arteries. They appeared to be a more healthy lutein cell. With fat stains the lutein cells were seen to be crowded with fatty substances.

Most of the fat was in the form of small globules. With Nile blue the majority of it was colored blue. The tissue was loaded with doubly refractile bodies, crystals and needles of cholesterin.

Aside from the reactions found in the structure of certain organs, there was another manifestation of increased activity on the part of the body in combating the excess of cholesterin compounds in the blood. The intima of the aorta and pulmonary artery, as well as the endothelium of some of the smaller arteries and arterioles, had responded so that definite alterations could be made out in their structure. Of these structures the intima of the aorta presented the most pronounced change. The ten animals in our series received an amount of cholesterin varying from 8.12 grams to 76.72 grams over a period of from nineteen to one hundred and sixty-six days for the individual rabbit. Five of the ten rabbits presented fully developed fatty plaques in their aortas. There was a proliferation of several layers of the large foam cells in the intima which was accompanied by fibroblastic increase with splitting of the internal elastic layer and the development of new elastic threads. The large foam cells were quite frequently found interposed by proliferation between the muscle cells of the inner half of the media. In Sudan stained sections the cells were filled with globular lipid, most of which in Nile blue preparations was tinged blue. Many crystals and needles of cholesterin were seen. With polarized light the foam cells were observed to contain large numbers of doubly refractile bodies. There were many cholesterin clefts in the deeper intimal tissues. Klotz and myself have undertaken a more intimate discussion of the intimal changes of the aorta in another paper.

A very interesting condition was found in the pulmonary artery in two of our rabbits. One of these animals receiving 76.72 grams of cholesterin in olive oil during one hundred and sixty-six days showed fatty plaques extending into the larger branches of the pulmonary artery in the lung. The other rabbit was fed 61.6 grams of cholesterin in a sodium oleate cholesterin emulsion over a period of one

hundred and fifteen days and presented well developed fatty intimal change in the small ramifications of the pulmonary artery. On section of the lungs of this latter animal these areas were seen as milk-white dots scattered over the cut surface. Sections of these areas proved to be an intimal proliferation of the small arterioles, consisting of large foam cells like those observed in the fatty plaques of the aorta. The structure of these areas was identical with those seen in the aorta. Some of the plaques had developed to such a degree that the lumen of the vessel was almost occluded. Frequently several plaques growing from different points in the intima were noted in a vessel. In some of the arteries there was an advanced splitting of the internal elastic layer with the foam cells between the split fibers. The foam cells were also found placed between the muscle cells of the media. These areas of proliferation showed the individual cells to be loaded with doubly refractile bodies along with many crystals and needles of cholesterin. Well formed fatty plaques were found in the carotids, iliacs, and femorals. In a similar manner varying degrees of intimal proliferation were noted in the branches of the coronary and renal arteries. In the arteriolæ rectæ of the kidney there was a proliferation of cells similar to those described in the larger arteries. The lining cells of these small vessels grew to such an extent that they could be recognized in the gross as white radiating streaks in the pyramids and microscopically were seen to form large islands of cells in the interstitial tissue. They were engorged with doubly refractive lipoid and actively engaged in cholesterin storage. Again where proliferation had not occurred, the lining cells of small capillaries often contained doubly refractive lipoid globules. This was particularly noted in the lining cells of the capillaries of the heart muscle of a rabbit which had received forced cholesterin feeding.

The association of a number of organs with cholesterin metabolism has been rather widely investigated. This is more particularly true of the adrenal and liver. More stress

has been placed upon the part the adrenal has to do with cholesterol metabolism than any other organ. As early as 1882 Gottschau pointed out that in pregnancy there is an hypertrophy of the cortex of the gland. Albrecht and Weltmann, Hueck and also Landau have emphasized the increase of cholesterol and fat in the blood during pregnancy. Sternberg found that the essential feature of the enlargement of the cortex in pregnancy and cholesterol feeding was a storage of fat and lipoids. This author further indicated that the condition of hypertrophy depended upon a hypercholesterinemia and only indicated a storing function of the adrenal cells. That the adrenal acts as a depot for cholesterol has also been strongly supported by Landau, Hueck, and Rothschild. The last-named author in his work on extirpation of the adrenal found that the vital significance of the organ depended upon the fact that it was able to store cholesterol. Stewart was able to remove both adrenals in gravid animals without affecting them and noted that the life of these animals could be preserved a long time by injecting cholesterol into the blood. Herxheimer has cited the case of an animal lacking both adrenals, but having a small accessory adrenal which lived a long time under cholesterol feeding and presented marked alteration of its aorta. By starving cats Gardner and Lauder found that the cholesterol of the adrenal diminished during the starvation period while that of the blood still remained constant. In fasting animals Rothschild was of the opinion that the cholesterol increase in the vital organs depended upon increased cholesterol in the blood and this in turn upon an accentuated destruction of fat tissue. He further compared the results of his suprarenectomy experiments with what he observed in fasting animals and found that there was a hypercholesterinemia and hypercholesteatosis in both instances. Along with these conditions there was a loss of body weight with a melting down of fatty tissue. With the development of a hypercholesterinemia in these experiments Rothschild also found that the liver played a very important part in protecting the

body against too great an accumulation of cholesterin in the blood by secreting an increased amount of cholesterin in the bile. Weltmann and Biach have demonstrated that carnivorous animals are more protected than herbivorous animals on account of the uniform secretion of cholesterin in the bile independent of conditions producing a hypercholesterinemia. However, Weltmann and Biach have also pointed out that herbivorous animals cannot excrete all of the cholesterin administered and it is consequently stored in their various organs. These authors have found an increase of cholesterin compounds in the liver cells, while Anitschkow and Chaladow have also noted a morphological and chemical excess of this material in the liver in feeding experiments.

Concerning the changes noted in other organs we find that Anitschkow and Saltykow have described the fatty plaques which occurred in the aorta of cholesterin-fed rabbits. In connection with suprarenectomy experiments Rothschild described the active part played by the Kupffer cells of the liver in dealing with cholesterin materials. According to Aschoff and Landau, the endothelial tissue of the spleen, lymph nodes, and bone marrow, together with the adrenal and Kupffer cells of the liver, constitute a very important intermediary apparatus in cholesterin metabolism. Anitschkow has called attention to the proliferation of large cells in the spleen and in his feeding experiments has described the accumulation of cholesterin compounds in the intima of aorta, spleen, and lymph nodes.

From a study of our findings we were particularly impressed with the fact that the organs of our animals contained large amounts of cholesterin free or combined. The adrenal and liver were very active in this respect and had accumulated so much of this material that the cells frequently presented signs of being taxed beyond their capacity. In the liver the Kupffer cells became filled with these materials very early. As the process of feeding advanced the amount of cholesterin in these organs always increased. In forced cholesterin

feeding the endothelium of the small capillaries and the endothelium of the arterial sinuses of the spleen respond in a marked degree along with the adrenals and liver. As the feeding is continued over a longer time other endothelial tissues respond, as that of the arteriolæ rectæ of kidney, intima of aorta and its branches as well as the pulmonary artery and its finer ramifications. Thus we were impressed by the response on the part of the body of some of its individual tissues. As each succeeding organ is called upon and becomes inadequate to care for the cholesterolin load, a time is reached when the equipment of the particular tissue is inadequate to satisfy the demand. Proliferation now occurs in the various organs as well as in the lining cells of the arteries and small capillaries.

As the amount of cholesterolin gradually increases within the body, the demand for greater assistance in caring for this material becomes imperative. The liver and adrenals comprise the most important organs first called on. When the work becomes too heavy for them, they are assisted by the corpora lutea, spleen, and endothelium of the blood vessels. Thus we are of the opinion that the alterations observed in hypercholesterinemia constitute a sequence of compensatory acts on the part of the body in an attempt to rid the blood of an excess amount of cholesterolin which cannot be properly excreted.

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THE MAINTENANCE OF VIRULENCE OF BACILLUS
ABORTIVUS EQUINUS.*

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Many pathogenic bacteria, when grown under artificial conditions, lose much of their virulence unless this is increased by passage through an animal body, by inserting the organism in an animal body for a time in collodion sacs, or by the addition of animal fluids to the culture medium. This decrease depends "particularly upon the selection, in artificial growth, of the less virulent or vegetative forms which grow actively and soon exceed in number their more pathogenic fellows. Each time the culture is transplanted more of the vegetative and fewer of the pathogenic micro-organisms are carried over, until finally the pathogenic bacteria are entirely eliminated or their virulence totally destroyed, and the entire culture is composed of vegetative or harmless forms of bacteria."¹ A number of instances can be mentioned in which the virulence of organisms is decreased by artificial culture, among the most common of which are *Bacillus paratyphosus* A,² whose virulence is lost somewhat readily in culture, *Pasteurella Gallinæ*,³ which dies rapidly in culture, *Bacillus Pestis*,⁴ which diminishes rapidly in artificial culture. "Scrapings of buboes sown on agar yield colonies varying in virulence (Yersin); the larger are only slightly virulent and grow so much more rapidly than the virulent colonies that the latter soon become crowded out with the result that subsequent sub-cultures rapidly lose their virulence." In artificial cultivation the pneumococcus soon loses its virulence and even its vitality.⁵ "Tubercle bacilli when just isolated from the mammalian body are usually quite virulent, but grow feebly on artificial culture media; after some months' cultivation they grow more luxuriantly, but have lost in virulence."⁶

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In the Animal Husbandry research laboratory of the Kentucky Agricultural Experiment Station there are quite a number of strains of *Bacillus abortivus equinus*, which have been grown under artificial conditions for varying lengths of time. These strains have been grown only on ordinary beef broth agar and have been transferred on an average of once every month and a half. The cultures have been kept in the lower part of the refrigerator at a temperature ranging from 8° to 12° C.

From observation on other microorganisms it might be inferred that the virulence of these cultures was so greatly reduced that they would be worthless in animal inoculation experiments. These cultures have not been handled in any way to increase their virulence, such as passing through an animal body, etc., and only strains were used for inoculation that were isolated from cases of abortion of mares reported to the laboratory. We have on hand several foreign strains of this organism which were not used in our experiments since there has been a great deal of controversy as to the characteristics and identification of this organism. We might mention at this point that a thorough search of the literature reveals nothing in regard to the retaining of virulence of *Bacillus abortivus equinus*, and relatively little work has been done with this important organism, due, no doubt, to its comparatively recent discovery.

The strains used in our experiments with the various animals are as follows:

A,	isolated	June,	1911,	from an	aborted	fetus	from a	jennet.
B,	"	Nov.,	1911,	"	"	"	"	" mare.
C,	"	Jan.,	1912,	"	"	"	"	" " "
D,	"	Feb.,	1913,	"	"	"	"	" " "
E,	"	Feb.,	1913,	"	"	"	"	" " "
F,	"	Feb.,	1913,	"	"	"	"	" " "
G,	"	March,	1913,	"	"	"	"	" " "
H,	"	Dec.,	1913,	"	"	"	"	" " "

In making the inoculations, mixed cultures were used exclusively, the cultures containing sometimes a few and sometimes all of the strains above. Twenty-four-hour old

broth cultures or twenty-four-hour cultures washed from agar slants were used in the experiments.

As has been mentioned above, these cultures were transferred on an average once every forty-five days. The average number of times for the transfer of the cultures then is approximately twenty, being thirty times for the oldest culture and ten times for the youngest culture. Very often in the experiments only the older cultures were used.

The animals used in these experiments were as follows: One mare, two rabbits, and two guinea-pigs. The mare was one which was used to draw a spring wagon on the Experiment Station farm. Two years previous to the experiment she had aborted, caused from an intravenous inoculation of *Bacillus abortivus equinus*. The following year she gave birth to a living, healthy foal. She was again bred on Nov. 13, 1914, and was in the best of health. On June 6, 1915, a blood sample was taken from this mare and tested for *Bacillus abortivus equinus* antibodies by means of the agglutination and complement fixation tests. None were present, and from this fact it was evident that all immunity conferred by the previous inoculation with the organism had disappeared. On July 16, 1915, this mare received one cubic centimeter of a mixed culture of *Bacillus abortivus equinus*, washed from an agar slant diluted in five cubic centimeters of physiological salt solution, intrajugular. This culture was of such a density as to be just about opaque and contained approximately 15,000,000 organisms. In connection with this inoculation two hundred cubic centimeters of a hyper-immune *Bacillus abortivus equinus* serum were injected subcutaneously.

After having been inoculated this mare was returned to the stall in which she had been kept for several months previous and was not allowed to run with other stock. It is highly improbable, therefore, that she could have become infected from an outside source.

After inoculation the mare's temperature gradually rose until it reached 104° F. on the third day, and then fell again to normal on the sixth day. For the first two days and a

half she would not touch food, but after this she began eating and all her symptoms were normal.

On July 29, 1915, the mare aborted a well-developed fetus, showing the typical symptoms and lesions of an abortion caused by the above organism. The characteristic "chocolate colored" discharge appeared, and all the organs of the fetus showed that the abortion had been caused by the germ. Plain agar plates were made from the afterbirth and organs of the fetus and the injected organism was recovered in every case. The mare was considerably depressed for two days after aborting and needed the services of a veterinarian, but after that time she rapidly recovered and was soon in perfect health.

From previous experience we have learned that the incubation period of this organism is from ten to fifteen days, the period in this case being thirteen days. It was striking to note the depressing effect such a relatively small dose of the culture had on this mare and to see that even after many generations' growth in the laboratory the organism would still produce a typical abortion.

Rabbit No. 129 received one-tenth cubic centimeter of a twenty-four-hour broth culture of *Bacillus abortivus equinus* in the marginal ear vein on Jan. 13, 1915. This culture was composed of the strains A, B, C, E, and G. The temperatures of the rabbit are interesting. On January 13th, at 10.30 A.M., before inoculation, the temperature was 102.2° F.; 11 A.M., 104.8° F.; 12 M., 104° F. The animal appeared listless and uncomfortable, eyes watering and conjunctiva exceedingly inflamed, breathing accelerated. Temperature 2 P.M., 104.2° F.; 5 P.M., 102.2° F. January 14th, temperature 8.15 A.M., 105.2° F.; 3 P.M., 105.6° F.; eating only a very little. January 15th, not eating at all; both eyes affected, right one nearly closed. Temperature 8.30 A.M., 104.6° F.; 2 P.M., 104.4° F. January 16th, condition unchanged, except rabbit seemed weaker. Had not eaten at all. Temperature, 8.30 A.M., 100.4° F. This temperature is the lowest we have recorded for a rabbit under any conditions. January 17th, animal died about 1 A.M. The

body was opened aseptically and plates were made of the heart, lungs, liver, spleen, kidneys, and stomach contents. Pure cultures of *Bacillus abortivus equinus* were obtained from the liver, spleen, kidneys, and heart.

Rabbit No. 146, on May 18, 1915, received one-tenth cubic centimeter of a twenty-four-hour mixed broth culture of *Bacillus abortivus equinus* in the marginal ear vein. This rabbit ran about the same course as described above and died on May 20, 1915. The fact that the dose killed more rapidly in this case might have been due to the difference in the vitality of the two animals, No. 129 being heavier and stronger than No. 146.

Pregnant guinea-pig No. 77 received, on May 18, 1915, one cubic centimeter of a twenty-four-hour mixed broth culture of the *Bacillus abortivus equinus*, subcutaneously. This pig lost sixty-four grams in three days, and on May 22d aborted four fetuses $8 \times 2\frac{1}{2}$ centimeters weighing fifty-seven grams. There was an abundant bloody discharge from the vagina. The afterbirth was not found, and the fetuses were partly eaten. Plates were made from the heart of the mother pig (which was killed), vagina, both horns of the uterus and from the fetuses. The organism was recovered from the fetuses.

Pregnant guinea-pig No. 195, on May 22, 1915, received one cubic centimeter of a mixed twenty-four-hour broth culture of *Bacillus abortivus equinus*, subcutaneously. This pig received five cubic centimeters of a hyperimmune *Bacillus abortivus equinus* serum subcutaneously on the same date. A deep subcutaneous abscess formed at the seat of the inoculation which healed slowly. The pig aborted four fetuses June 8, 1915, $9 \times 2\frac{1}{2}$ centimeters. The animal was killed and plates were made from uterus, vagina, and heart's blood. The organism was obtained from the uterus.

SUMMARY.

The intravenous inoculation of a mare with one cubic centimeter of a mixed culture of *Bacillus abortivus equinus* which had been grown in the laboratory for from ten to

thirty generations and for from one and one-half to four years, caused a typical abortion in a mare whose blood showed no immunity to the disease. This mare at the same time was protected with two hundred cubic centimeters of a hyperimmune serum which had very remarkable bacteriolytic properties. The amount of *Bacillus abortivus equinus* culture injected in this instance was less than half the amount formerly used in this laboratory in causing abortion in mares experimentally.

The intravenous inoculation of one-tenth of a cubic centimeter of twenty four-hour broth cultures produced death in rabbits in from two to four days, and the subcutaneous inoculation of one cubic centimeter of a broth culture produced abortion in pregnant guinea-pigs in four days and seventeen days. The organism producing these effects was recovered in each instance.

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AN UNIQUE LESION OF THE HEART IN SYSTEMIC BLASTOMYCOSIS.*

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A review of those cases of systemic blastomycosis in which post-mortem examinations were made emphasizes the wide range and the multiplicity of infection-sites in this condition. The skin, lungs, and bony structures are most frequently involved, but there seem to be few organs or tissues which are not subject to invasion. Whether the primary site of infection in these cases is usually pulmonary, as held by Stober¹ and others, or cutaneous as originally held by Busche² and recently stated by Mallory,³ need not be considered in this connection. Whichever of these views is correct, the lesions in the deeper organs are necessarily metastatic. The organs which at autopsy have been found to contain these metastases from skin or lung are, in order of frequency, bone, spleen, liver, brain, and meninges, lymph nodes, kidneys, pleura, prostate, pancreas, heart, pericardium, spinal cord, epididymis, eye, larynx, appendix, and testicle. This is based on a tabulation of twenty-one complete post-mortem examinations. In but one case of this series was the lung found uninvolved, and in but one, similarly, was the skin free from lesion at time of autopsy. The reported instances of cardiac involvement are very few. Cleary⁴ reported a case in which, though there was no gross evidence of invasion of the heart, histological examination showed lesions containing the blastomycetes in the myocardium. In a case reported by Churchill and Stober⁵ the organism was said to have been recovered by culture from the pericardial fluid. In this instance, however, neither macroscopic nor microscopic examination revealed any lesion of the heart muscle. LeCount,⁶ in a paper as yet unpublished, reported

* Read in abstract before the American Association of Pathologists and Bacteriologists, St. Louis, Mo., April 2, 1915. Received for publication Nov. 8, 1915.

a case which he described as miliary blastomycotic retrogressive lymphangitis of the epicardium, arising from lesions in the bronchial lymph nodes. In this, however, there was no involvement of the myocardium.

The specimen to be described is, so far as a search of the English literature of the subject shows, unique on account of the size, number, and location of the heart lesions and because of certain unusual features in their gross appearance. The complete report of this case will be published elsewhere, in a study of collected cases of systemic blastomycosis. Briefly, the essential points were as follows:

M. N., a German laborer, age 61, was admitted to the Charity Hospital May 14, 1914. He was troubled by a cough which had persisted for three months. A diagnosis of pulmonary tuberculosis was made, though sputum examinations were negative. There was evidence of cardiac disturbance, but this was a minor feature. The most distressing symptoms were cough and dyspnea, thought to be due to bronchial asthma. Patient was later transferred to a surgical service for treatment of an abscess of the scalp. He became progressively worse but deserted in August.

In November he returned in very poor condition. Sputum and Von Pirquet test were both negative. Several extensive abscesses and bone lesions were present. Death, which occurred January 16, 1915, was apparently due to the pulmonary condition with a terminal septicemia.

The autopsy (A-15-25) was performed on January 18, 1915, forty hours post-mortem. Blastomycotic lesions were found in the skin of the face, scalp, chest, and arm; in the bones of the skull, chest-wall, and elbow and wrist joints on the right arm, as well as in both lungs, the spleen and the cerebrum and its meninges. The subcutaneous abscesses of the chest wall seemed to arise from the underlying necrotic ribs. From the lower abscess a sinus continued inward through the fifth intercostal space and was found to communicate directly with the pericardial cavity. The pericardial cavity contained very numerous dense fibrous adhesions between the thickened pericardium and the heart, among which was much fibrin and many collections of purulent material. The largest of these was in communication externally with a subcutaneous abscess and internally with a necrotic area in the anterior wall of the left ventricle of the heart.

Description of specimen.—The heart (Fig. 1) is very considerably larger than normal and weighs 420 grams. As a whole, it is a light reddish brown in color, though its surface shows several grayish yellow areas, necrotic at the center, irregular and sometimes indefinite in outline, though usually well demarked and surrounded by zones of congestion. These lesions vary from 1 to 3 centimeters in diameter and appear to extend for variable distances into the myocardium. One soft necrotic area in the

anterior wall of the left ventricle measures roughly 3 centimeters in diameter and is found to extend into the greatly hypertrophied wall for a distance of 1.5 centimeters. Another similar lesion in the posterior wall of the right auricle is seen on section to extend through the muscularis and involve the endocardium (Fig. 1, a).

Upon opening the right auricle many minute pearl gray to white nodules (Fig. 1, b) are seen on the inner surface overlying two areas of myocardial involvement. One of these lesions is that cut through in opening the chamber, the other is in the inter-auricular wall. Some of the small nodules seem entirely subendocardial and are clearly visible through this tissue, while others have penetrated it and protrude into the auricular cavity for distances of 1 to 2 millimeters. Numbers of these small lesions of the endocardium present the appearance of relatively deep, crater-like depressions surrounded by thin, shell-like grayish, semi-translucent walls. These tend to slope upward in the form of a cone, and with the central depression leading to more or less soft necrotic tissue present the appearance of craters which would discharge necrotic, semi-purulent material directly into the blood stream. Those papular nodules which have not developed craters are more opaque on account of this necrotic material which lies immediately beneath the surface.

In the inter-auricular septum is a large lesion which projects considerably into the right cavity (Fig 1, c). The endocardium in this region shows many small nodules, and a few crater-like eminences similar to those described above. These lead to extensive necrotic lesions in the inter-auricular wall. In the left auricle this lesion is apparent, but is not so prominent, and no crateriform openings can be found. There is no endocardial involvement in either of the left chambers.

Microscopically sections of the tissue removed from the anterior wall of the left ventricle show lesions typical of blastomycotic infection (Fig. 2). At this point the condition extends one-half the distance through the myocardium. The older parts of the lesion show extensive necrosis together with much acute inflammatory exudate. The deeper and more recent lesions are, except for a varying degree of polymorphonuclear invasion, very suggestive of tuberculosis, showing endothelial proliferation, lymphoid and plasma cell infiltration, the formation of giant cells and central necrosis. The larger lesions appear to be made up by the fusion of the small foci. Within many of the giant cells, as well as free among the infiltrating cells of inflammation, are more or less numerous blastomycetes of the ordinary sclerotic type seen in tissue lesions. Relatively few present the appearance of budding.

CONCLUSION.

The specimen described is a remarkable instance of mycotic invasion of the myocardium. The only other reported case of blastomycosis of the musculature of the heart, directly or indirectly discoverable in the literature, in no way compares with it in location or extent of the lesion.

The communication by a sinus between the pericardial cavity and the body surface, the number and extent of the blastomycotic lesions in the outer part of the heart muscle, together with the inter-auricular focus and the numerous minute endocardial nodules and sinuses, many of which had evidently been discharging directly into the blood stream of the right heart, make the specimen presented unique.

It may be noted that the lesions in the lungs, seeded by the blood stream, were extremely numerous and, for the most part, very small.

[I wish to thank Dr. H. W. Wade for photographs and for other assistance.]

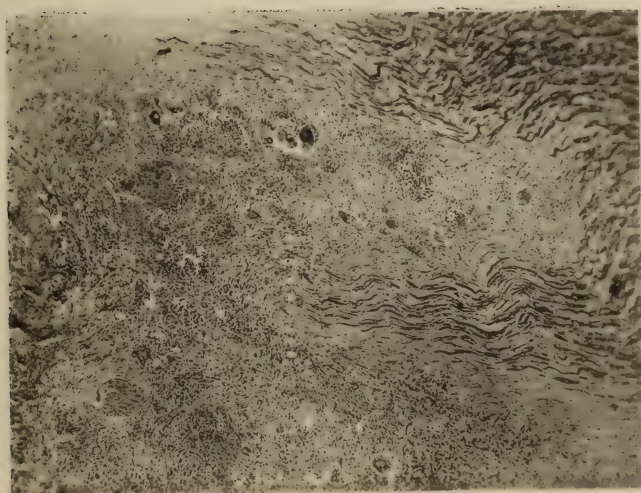
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DESCRIPTION OF PLATE XIX.

FIG. 1. — Heart, photographed from behind and to the right. Lesion (a) of posterior wall of right auricle extending entirely through the muscularis. A similar focus (c) in the inter-auricular wall. Numbers of papular, sometimes crater-like nodules (b) arise from the deeper layers and involve the endocardium.

FIG. 2. — Photomicrograph of section from lesion on anterior surface of left ventricle, illustrating the invasion of the muscularis and the typical blastomycotic nature of the lesion.



THE EFFECT OF TEMPERATURE UPON THE CLOTTING TIME
(PROTHROMBIN TIME) OF OXALATED PLASMA WITH
CALCIUM.*

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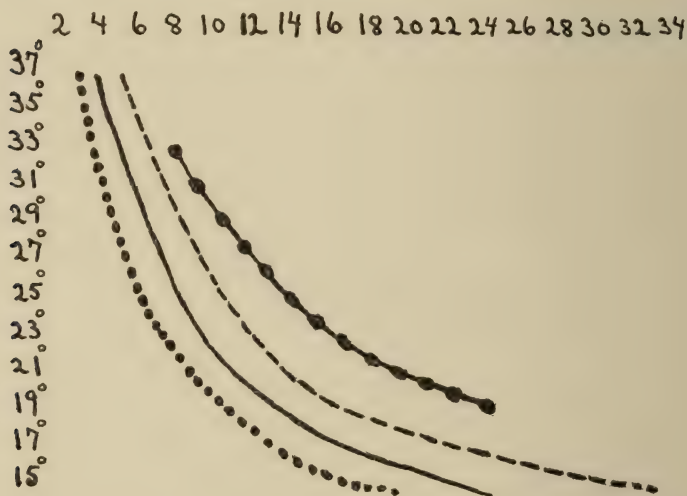
The clotting time on recalcification of oxalated plasma has been used by Howell¹ as a test for the relative efficiency of prothrombin. This clotting time on recalcification has been called prothrombin time² and in comparing two such times, the longer is taken to represent a less amount or relative deficiency of prothrombin, the shorter the reverse. Howell¹ found that it took eight to thirteen minutes, averaging ten, for normal human plasma (obtained by drawing blood from a vein with a syringe and mixing it at once with .1 per cent sodium oxalate in .9 per cent salt solution and centrifugalizing at about 3,000 revolutions a minute for twenty minutes) to form a solid clot with the optimum amount of calcium, that is the amount that causes the most rapid clotting.

Minot and Denny² studied this test and found in ninety-one determinations on sixty-six normal persons variations of from six to fourteen minutes (tested at room temperature). They found that when a series of normal plasmas were tested at the same time there was a distinct tendency for the prothrombin times to be grouped in a high, medium or low range — that is, one would see variations of ten to fourteen, six to eight or ten, and eight to twelve; only once did six (it is believed that this case had had no food for over eighteen hours) and fourteen occur at the same time. They suggested that from figures alone one could not tell if six or fourteen was abnormal, but if one had a series of normals clotting in six or eight minutes and a plasma from a patient tested at the same time clotting in twelve or fourteen minutes, one would be inclined to believe he was dealing with a pathological condition. Temperature seemed to be a partial cause for the variation of the prothrombin time.

* Received for publication Nov. 10, 1915.

That this is true is shown by a study of how temperature affects this reaction.

Many writers have shown that the coagulation time of the whole blood varies with temperature, being longer the lower the temperature; the amount that it is affected varying with the different methods of testing coagulation time. Howell³ has pointed out that the action of antithrombin is greatly augmented at body temperature and high temperatures weaken thrombin. It is thus only natural that temperature affects the prothrombin time as is shown in the following chart:



The horizontal figures represent the clotting time in minutes and the vertical figures degrees centigrade.

The solid line represents the average prothrombin time of ten normal plasmas tested at varying temperatures.

These were tested in March and April, 1915. The plasmas were obtained by the method described above. Plasmas from normal individuals obtained by drawing blood directly into oxalate solution showed similar though perhaps not quite so wide variations as when the blood was rapidly drawn and then mixed at once with oxalate. This may be because a very small amount of thrombin may be formed during the collection of the blood by this latter method, as has been pointed out in a paper on the effect of chloroform upon the factors of coagulation.⁴

The tests as in previous work were done shortly after the plasma was obtained because, as pointed out before,² allowing plasma to stand may vary its prothrombin time.

Two of these plasmas tested at the same time at room temperature 21° C. showed the widest variation seen by Minot and Denny for normal plasmas tested at the same time. The dotted and dashed lines show the prothrombin times for these two plasmas when tested at different temperatures. These two plasmas are taken to represent the maximum and minimum normals. The beaded line shows an abnormal prothrombin time obtained in a case of pneumonia. The chart shows that the lower the temperature the longer the prothrombin time. There is a gradual lengthening of this time between 37° C. and 22° C. Below this latter temperature the prothrombin time lengthens rapidly as the temperature falls, thus making it undesirable to do the test when the plasma is below 21° C.

These curves given for the coagulation of oxalated plasma with the optimum amount of calcium at different temperatures are not unlike the observations upon the coagulation of the whole blood.

If one takes a sample of oxalated plasma and allows a part to be chilled (11° C.) and a part to be kept warm (35° C.) and then brings both to the same temperature (22° C.), the one that has been chilled will clot on recalcification slightly slower than the one that has been warmed. Of course if both warmed and cooled specimens without being brought to the same temperature are placed at the same room temperature the cooled one will clot slower than the warmed one. When plasmas were tested in warm weather at the same temperature as in cold, one obtained the impression that in warm weather they usually clotted slightly faster than in cold. Perhaps humidity played a part.

Though temperature plays a part in the variation of the prothrombin time, there is, however, a normal variation of five minutes (9 to 14 minutes) when tested at 21° C. To compare tests done on different days it is desirable that the

test be made at the same temperature, preferably about 22° C., a temperature at which the accurate coagulation time can be conveniently detected. It is, however, desirable when testing a pathological case to always run at the same time a control for comparison, as has been pointed out in the papers previously mentioned and also by Hurwitz and Drinker.⁵

If a plasma when tested is above 21° C. and under 27° C. and gives a prothrombin time of over fourteen minutes or under six minutes, or a prothrombin time of five minutes longer or shorter than the control one, then it definitely has an abnormal prothrombin time. However, an abnormal prothrombin time may occur which is not five minutes longer or shorter than the control, as may be seen by referring to the chart and from the following example: If the test was done at 25° C. and the control was the maximum normal, it would give eleven minutes, while the pneumonia case which has an abnormal prothrombin would give fourteen minutes. If tested at 22° C. the same control's prothrombin time would be thirteen minutes and the case of pneumonia would have a definitely abnormal prothrombin time, nineteen minutes. Thus to be sure of detecting an abnormal prothrombin time it is best to do the test at about 22° C.

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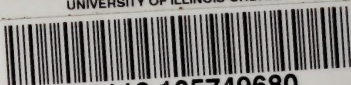
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